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Nondisruptive Detection of Activity of Catabolic Promoters of *Pseudomonas putida* with an Antigenic Surface Reporter System

ÁNGEL CEBOLLA,[1] CARLOS GUZMÁN,[2] AND VÍCTOR DE LORENZO[1]*

Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid 28006, Spain,[1] and National Center for Research in Biotechnology, Braunschweig, 38124, Germany[2]

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A simple procedure to detect the switching on and off of catabolic promoters of *Pseudomonas putida*, at the level of single cells based on the immunodetection of a reporter epitope expressed on the surface of bacterial cells, has been developed. To do this, the antigenic sequence Asp-Leu-Pro-Pro-Asn-Ser-Asp-Val-Val-Asp, from a coronavirus, was inserted genetically in the permissive site around amino acid position 153 of the LamB protein (maltose and lambda phage receptor) of *Escherichia coli*. When the hybrid *lamB* gene is transcribed, the epitope becomes presented on the surface of the bacterial cells in a configuration available to specific antibodies. To validate this notion in nonenteric bacteria, the expression and correct processing of LamB were confirmed by coupling the *lamB* gene to the salicylate-responsive *Psal* promoter of the NAH7 (naphthalene degradation) plasmid in *Pseudomonas putida*. Subsequently, a hybrid *lamB* gene carrying the sequence of the coronavirus antigen was placed downstream of the *m*-toluate-responsive *Pm* promoter of the TOL (toluene degradation) plasmid. Exposure of the epitope on the *Pseudomonas* cell surface was monitored through fluorescence of whole cells treated with a monoclonal antibody against the heterologous antigen. Fluorescence emission was dependent on the presence of *m*-toluate in the medium, thus permitting detection of the *Pm* promoter switching on by simple optical inspection of individual cells, even in situations when these are a very minor component of a complex bacterial community.

Since the pioneering use of β-galactosidase for monitoring the activity of the *lac* system in *Escherichia coli* (1), the use of reporter genes has been at the basis of nearly every study on gene regulation in bacteria. A whole variety of genes encoding enzymatic, optical, immunological, and physical markers, many of which have been used for studying specific promoters in response to various environmental signals, are currently available (8). For the most part, however, studies on bacterial gene expression are done under defined physicochemical conditions, in monocultures, and with a very limited number of variables. The information obtained on promoter activity under such circumstances may have little to do with the actual performance of the same promoter in an open, i.e., natural environment, where the number of variables increases and the final outcome of transcriptional activity may be determined by signals very different from those identified in the laboratory (11). One archetypal instance of this is the regulation of catabolic promoters of biodegradative pathways of *Pseudomonas* species, such as those encoded by the TOL (toluene degradation) and NAH (naphthalene degradation) plasmids (24, 30). Although the regulation of the various promoters involved in the expression of the corresponding catabolic operons has been studied in the laboratory in great detail, it has not been possible so far to determine their performance in complex environments like those where this type of bacteria resides naturally. Understanding how promoters are regulated in situ may also be important if the regulated functions are to be used in bioremediation of contaminated sites (11). In addition, bacteria frequently enter a nonculturable state (28), which renders useless most reporter systems, for which the cells under study must be grown in culture.

In recent years, the use of fluorescent substrates of β-galactosidase, along with digitized video microscopy (22, 23), has permitted the visualization of specific gene expression in *Salmonella* and *Bacillus* species at the level of single cells. Unfortunately, fluorogenic β-D-galactopyranoside derivatives are eventually diffusible and their transport through the membranes of many bacteria of environmental interest is quite unpredictable. Furthermore, many microorganisms present in natural environments have an intrinsic β-galactosidase activity, which limits the application of this reporter system to a reduced number of instances. An alternative approach has included the use of *Vibrio harveyi* or *Vibrio fischeri* luciferase (*lux*) or firefly luciferase (*luc*) genes, determining a phenotype of light emission strong enough to allow its detection in legume nodules containing labeled rhizobia (4, 21). Detection of light emission by single cells requires, however, ultrasensitive charge-coupled device cameras (33) of photon-counting setups. Thus far, this has allowed detection of individual cells of root-colonizing *Pseudomonas* species when the reporter *lux* genes are placed downstream of a strong promoter in multicopy gene dosage (31). The practical utility of *lux*-based systems for monitoring promoter activity in situ remains to be assessed.

In this work, we have sought to develop a reporter system for monitoring the switching on of catabolic promoters of individual *Pseudomonas* cells in the complex environments where biodegradative pathways are expected to perform. The basis of such a system is the genetic coupling of promoter activity to the presentation of a strongly antigenic sequence from the transmissible gastroenteritis coronavirus (TGEV) on the surface of *Pseudomonas* cells in a conformation amenable to specific recognition by monoclonal antibodies (MAbs). This approach has

* Corresponding author. Mailing address: Centro de Investigaciones Biológicas (CSIC), C/Velázquez 144, 28006 Madrid, Spain. Phone: (341) 5611800. Fax: (341) 5627518.

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TABLE 1. Bacteria, plasmids, and transposons used in this work

Strain or plasmid	Relevant genotype and characteristics	Reference or origin
E. coli K-12		
CC118	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA thi-1 rpsE rpoB argE*(Am) *recA1*	16
CC118λ*pir*	CC118 lysogenized with λ*pir* phage	16
S17-1λ*pir*	Tp^r Sm^r *recA thi hsdRM*$^+$ RP4::2-Tc::Mu::Km Tn*7*, λ*pir* phage lysogen	12
CC118 F′SURE	CC118 F′::[Tc^r, *lacI*^{q}ZΔM15]	Lab collection
POP6510	*thr leu tonB thi lacY recA dex-5 metA supE*, frameshift mutation in *lamB*	2
POP6510-126A	POP6510 with chromosomal insertion of mini-Tn*5 xylS/Pm→lamB-A6*	This work
P. putida		
KT2442	Prototophic, Rif^r derivative of KT2440	16
KT2442-41W	Km^r, KT2442 with chromosomal insertion of mini-Tn*5 nahR/Psal→lamB*	This work
KT2442-116A	Km^r, KT2442 with chromosomal insertion of mini-Tn*5 xylS/Pm→lamB-A6*	This work
Plasmids		
pAC1	Ap^r, *oriV* ColE1, *lacI*$^{q+}$, *lamB*$^+$ expressed through *Ptac* promoter	2
pAJC264	Ap^r, same as pAC1, but the *lamB* sequence has a *Bam*HI site at structural codon 153, (*lamB-153*)	2
pCOR264	Ap^r, same as pAJC264, but sequence of TGEV epitope inserted at its single *Bam*HI site, *lamB-A6*$^+$	This work
pVDL8	Cm^r, low-copy-number vector, *oriV* pSC101, derivative of pHSG575 (35) with the same polylinker as p18Not (16)	This work
pLBB9	Cm^r, pVDL8 inserted with the promoterless *lamB-153* sequence of pAJC264 as a 1.4-kb *Eco*RI-*Hin*dIII fragment flanked by *Not*I sites	This work
pLBB9-A6	Cm^r, pLBB9 with sequence of TGEV A6 epitope inserted at its single *Bam*HI site; promoterless *lamB-A6* sequence as a *Not*I cassette	This work
pCNB1	Ap^r Km^r, R6K*oriV* RP4*oriT*, delivery vector for mini-Tn*5 xylS/Pm*	12
pTCOR12	Ap^r Km^r, pCNB1 inserted with the promoterless *lamB-A6* sequence in front of *Pm* promoter as a *Not*I cassette; delivery vector of mini-Tn*5 xylS/Pm→lamB-A6*	This work
pCNB4	Ap^r Sm^r, R6K*oriV* RP4*oriT*, delivery vector for mini-Tn*5 nahR/Psal*	12
pCNB4-*lamB*	Ap^r Sm^r, pCNB4 inserted with the promoterless wt *lamB* sequence in front of the *Psal* promoter as a *Not*I cassette; delivery vector of mini-Tn*5 nahR/Psal→lamB*	This work

permitted us to visualize, through immunofluorescence, the activity of the *m*-toluate-responsive *Pm* promoter of the TOL plasmid pWW0 in individual cells of *Pseudomonas putida* inoculated into the activated sludge of an oil refinery.

MATERIALS AND METHODS

Strains, plasmids, media, and general procedures. Relevant bacterial strains, plasmids, and transposons used in this work are listed in Table 1. Lambda phages λh$^+$, λh^0, and λhh* have been described elsewhere (3, 6). *E. coli* CC118 F′SURE was used as the recipient of most plasmid constructions. Plasmids containing an R6K origin of replication were kept in *E. coli* CC118λ*pir*. *E. coli* S17-1 λ*pir* was used as the mobilizing strain for *oriT*RP4-mediated conjugal transfers (13). Minimal M9 medium (with 0.2% citrate as the only carbon source) and complete Luria-Bertani medium (LB) (29) were supplemented, when required, with ampicillin (150 μg/ml), kanamycin (50 μg/ml), rifampin (50 μg/ml), or piperacillin (30 μg/ml). Recombinant DNA techniques were carried out by standard methods (29). For PCR, 50 to 100 ng of each the DNA templates was mixed with 50 pmol of each of the primers in a volume of 100 μl, with 5 U of *Taq* DNA polymerase added, and subjected to 30 cycles of alternate denaturing (1 min at 95°C), primer annealing (1 min at 48°C), and extension (2 min at 72°C). Hybrid transposons carrying various DNA segments were integrated into the chromosome of target *Pseudomonas* strains as previously described in detail (13).

Protein techniques. Whole-cell extracts were analyzed by electrophoresis in denaturing 12% polyacrylamide gels (20). When required, proteins were transferred onto Immobilon membranes (Millipore) by electroblotting of the corresponding gels (36). Blotted membranes were blocked with 2% skim milk in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, 3 mM KCl [pH 7.4]) for 30 min and then washed three times for 10 min each with the same buffer. Polyclonal rabbit serum raised against denatured LamB protein (a kind gift of M. Hofnung) was then added at a 1:4,000 dilution and incubated for 1 h, after which the blots were washed three times for 10 min with PBS buffer as above. The membranes were then incubated with 0.5 μg of protein A-peroxidase (Sigma) per ml, washed again with PBS for 15 min, and briefly rinsed with distilled water. The positions of the LamB and LamB-A6 proteins were revealed with 0.02% diaminobenzidine tetrahydrochloride (Sigma) and 0.03% oxygen peroxide. For detection of the coronavirus antigen, blocked blots were incubated with a 1:5,000 dilution of an ascites fluid containing the murine MAb 6A.A6 (Ingenasa) as mentioned above; the antigen was identified with a goat anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase (Sigma). A rapid screen for exposure of the TGEV antigen on the surface of *E. coli* and *Pseudomonas* cells was made by blotting the bacterial colonies growing on a petri dish directly onto circular Immobilon membranes for 2 min. The filters were lifted off the plate, sandwiched between two Whatman 3MM (Millipore Iberica) filter paper sheets wetted in PBS, and dried at 65°C for 30 min under vacuum. The membranes were then washed with fresh PBS, blocked with 2% skim milk, and incubated with antibodies as described above.

Construction of mini-Tn5 *nahR/Psal→lamB*. For expression of the wild-type (wt) LamB protein in *P. putida*, we placed the promoterless structural sequence of *lamB* (9) in front of the salicylate-responsive *Psal* promoter of the NAH7 (naphthalene biodegradation) plasmid as follows. Two PCR primers, 5′-GGGAATTCAGGAGATAGAATGATG-3′ and 5′-GTTTTAAGCTTACCACCAGATTTCC-3′, were designed to amplify the *lamB* sequence DNA from plasmid pAC1 (Table 1) while simultaneously introducing a single *Eco*RI site just upstream of its ribosome-binding site and an *Hin*dIII site downstream of its termination codon TAA (see Fig. 3). The resulting restriction fragment was cloned at the corresponding sites of pVDL8 (Table 1), which endows the *lamB*-containing DNA segment with flanking *Not*I sites. These were then used to produce a *Not*I fragment, which was cloned, in the proper orientation, at the single *Not*I site of pCNB4 (12), thus creating a delivery vector for hybrid transposon mini-Tn*5 nahR/Psal→lamB* (see Fig. 2). This mobile element was then inserted into the chromosome of *P. putida* KT2442 as described previously (13), giving rise to *P. putida* KT2442-41W.

Construction and expression of hybrid LamB-A6 protein. To construct a LamB derivative carrying the antigenic site C of glycoprotein S of TGEV, i.e., the A6 antigen, we designed a synthetic linker formed by the two synthetic oligonucleotides 5′-GATCTGCCCCCCAACTCGGACGTCGTC-3′ and 5′-GATCGACGACGTCCGAGTTGGGGGGCA-3′, encoding the amino acid sequence Asp-Leu-Pro-Pro-Asn-Ser-Asp-Val-Val-Asp. This linker was inserted as a short *Bam*HI restriction fragment at the same site of vector pAJC264 (Table 1). This resulted in plasmid pCOR264, which encodes a modified *lamB* sequence (*lamB-A6*) corresponding to a hybrid protein in which an extra 10 amino acids was inserted at position 153 of the polypeptide chain, followed by the rest of the protein. This construction was used for preliminary characterization of LamB-A6 in *E. coli*. For its expression in *Pseudomonas* species, the sequence was subjected to PCR and subcloned in the pVDL8 vector as described for the wt *lamB* gene

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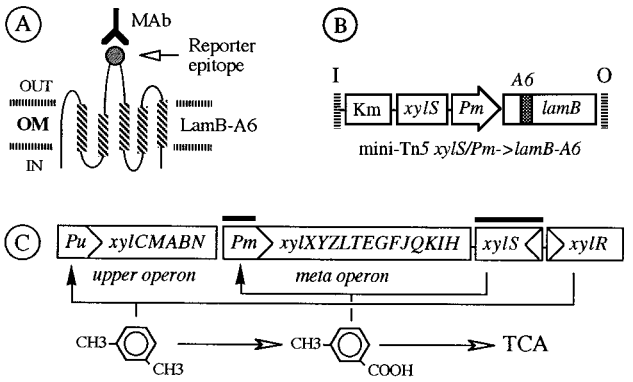

FIG. 1. Construction of a hybrid LamB-A6 protein as a surface reporter of promoter activity. (A) The LamB protein of *E. coli* contains about 12 outer membrane (OM)-spanning domains (symbolically represented in the figure) connected by various polypeptide loops facing the periplasm (IN) or the external medium (OUT). Amino acid 153 is located at one permissive loop (2, 7), which allows exposure of heterologous peptides (like the TGEV A6 epitope used in this work) on the cell envelope, where they can react in situ with specific MAbs. (B) The scheme shows the organization of the chromosomal insertion of mini-Tn*5* *xylS*/*Pm*→*lamB-A6* carrying relevant regulatory elements (not to scale) engineered in a transposon vector bounded by the I and O termini of Tn*5*. (C) The figure (not to scale) summarizes the major regulatory elements which control transcription of the TOL genes encoded by catabolic plasmid pWW0 for biodegradation of toluene and *m*/*p*-xylenes (24). The regulatory elements of the pathway included within hybrid transposon mini-Tn*5* *xylS*/*Pm* are indicated by solid bars above of the scheme of the TOL operons. TCA, tricarboxylic acid cycle.

(see above) to generate a *Not*I restriction fragment spanning a promoterless *lamB-A6* gene. Once obtained in plasmid pLBB9-A6 (Table 1), the fragment was cloned at the *Not*I site of pCNB1 (12), thus creating a delivery vector for hybrid transposon mini-Tn*5* *xylS*/*Pm*→*lamB-A6* (Fig. 1B). This places transcription of the *lamB-A6* sequence under the control of the *m*-toluate-responsive promoter *Pm* of the TOL (toluene degradation) plasmid pWW0. The mobile element was inserted into the chromosome of *P. putida* KT2442 as described previously (13), and clones containing different inserts were analyzed as discussed in Results.

Immunofluorescence microscopy. For analysis of epitope presentation on the surface of bacterial cells, 5-μl volumes of the samples under study (or an adequate dilution of them) were placed on the wells of a multitest glass slide (Flow Laboratories, Meckenheim, Germany) and fixed by briefly heating the slide on the flame of a gas burner. 4′,6-Diamidine-2-phenylindole dihydrochloride (DAPI) staining of whole bacterial populations was performed by adding to the fixed samples a minimal volume of a 1-μg/ml solution of the dye for 5 min and washing them with distilled water. Subsequently, each sample was treated for 1 h at room temperature with 5 μl of a 1:50 dilution of the ascites fluid containing the MAb 6A.A6 mentioned above (15) in PBS containing 10% fetal calf serum (GIBCO) or 1% bovine serum albumin (Boehringer Mannheim). Slides were then rinsed with PBS buffer and treated with fluorescein-labelled goat anti-mouse immunoglobulin G (Dianova, Hamburg, Germany). For this, 5 μl of the antibody, diluted 1:50 in PBS containing 10% fetal calf serum or 1% bovine serum albumin, was added to each sample and allowed to react with the first antibody for 1 h at room temperature. The slides were then washed twice in PBS, rinsed with distilled water, and air dried. Samples were examined under either phase-contrast or epifluorescence microscopy with a Zeiss Axiophot microscope. Phase-contrast images were captured with Agfapan APX 100 film, while immunofluorescence of bacterial cells was recorded with Ilford 400 Delta film.

RESULTS AND DISCUSSION

Rationale of the antigenic surface reporter system. LamB is the outer membrane protein of *E. coli* which serves as the receptor for maltose and maltodextrines as well as the port of entry of bacteriophage lambda (34). Previous genetic and structural studies on LamB have identified one specific location of the protein, spanning amino acid positions 153 and 154, which encompasses a loop of the polypeptide chain exposed to the external medium (2, 5, 7). This site tolerates the genetic insertion of up to 232 extra amino acids without loss of the overall folding pattern of the hybrid protein compared with the wt LamB (32). Shorter antigenic sequences can also be inserted in that permissive site, and they then become available to recognition by specific antibodies without any cell disruption (7). As shown in Fig. 1, we reasoned that if properly expressed, folded, and exposed in the heterologous gram-negative hosts, the LamB protein could be used as a universal carrier to endow the surface of bacterial cells of interest with a novel and very specific antigenic property suitable for physical in situ monitoring with antibodies. Furthermore, if expression of the hybrid protein is coupled to a promoter of interest, the antigenicity of the whole cell should reflect its transcriptional activity under the given environmental conditions. The following sections validate these notions experimentally.

Expression of the LamB protein of *E. coli* in *P. putida*. Since our final aim was to monitor catabolic promoter activity in *Pseudomonas* strains with biodegradative phenotypes, we first examined the expression and correct processing of native LamB in *P. putida* KT2442 (Table 1), which is one of the standard strains used as plasmid hosts for metabolism of aromatic hydrocarbons. Heterologous expression of functional LamB has been confirmed in various enterobacteria and *Vibrio* species (27) but not in *Pseudomonas* species (14). However, since expression assays in non-*E. coli* species were performed, for the most part, on the basis of sensitivity to lambda, the resistance to this phage does not mean necessarily lack of expression (26). To address this issue, we produced a promoterless wt *lamB* sequence, which was cloned in front of the salicylate-responsive *Psal* promoter of the NAH7 (naphthalene degradation) plasmid of *P. putida* (30, 39). As shown in Fig. 2, a DNA segment containing the promoter of interest in front of the *lamB* gene, along with the cognate regulator of the system, the *nahR* gene (17), and a kanamycin selection marker, was constructed. The whole unit was then placed in a mini-Tn*5* transposon vector and inserted into the chromosome of *P.*

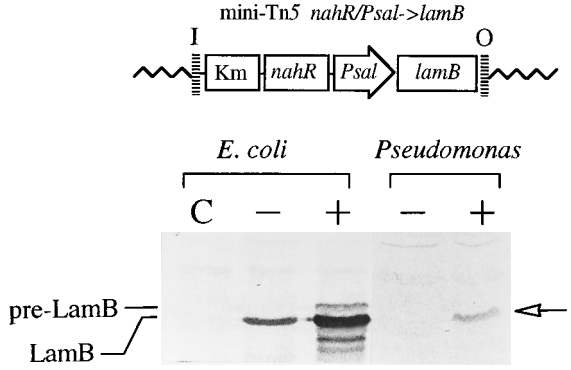

FIG. 2. Expression of LamB in *P. putida* KT2442. The organization of the hybrid mini-Tn*5* transposon assembled to examine the expression of wt LamB in *P. putida* is sketched at the top of the figure. The transposon was inserted into the chromosome of *P. putida* KT442, and expression of LamB was detected by Western blot analysis. Approximately 10^8 cells of each strain were run in a denaturing polyacrylamide gel, blotted on a membrane, probed with a 1:4,000 dilution of polyclonal rabbit anti-denatured LamB serum, and further developed with protein A conjugated to horseradish peroxidase, as described in Materials and Methods. The first three lanes are *E. coli* POP6510 (*lamB*) controls, either plasmidless (C) or transformed with *lamB*$^+$, isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible plasmid pAC1 (Table 1). Samples were taken from each of the *E. coli* cultures in Luria-Bertani medium at 30°C grown to an optical density at 600 nm of 0.5, and induced for 2 h with 1 mM IPTG (lane +). Lane − is an uninduced control which shows a considerable basal level of expression from the *Ptac* promoter of pAC1. The last two lanes are samples of *P. putida* KT2442-41W, with mini-Tn*5* *nahR*/*Psal*→*lamB* inserted. In this case, *Pseudomonas* cells were grown at 30°C in Luria-Bertani medium as above and induced for 5 h with 2 mM sodium salicylate (lane +) or left uninduced (lane −). The positions of the mature LamB protein and the unprocessed product (pre-LamB) are indicated to the left of the gel.

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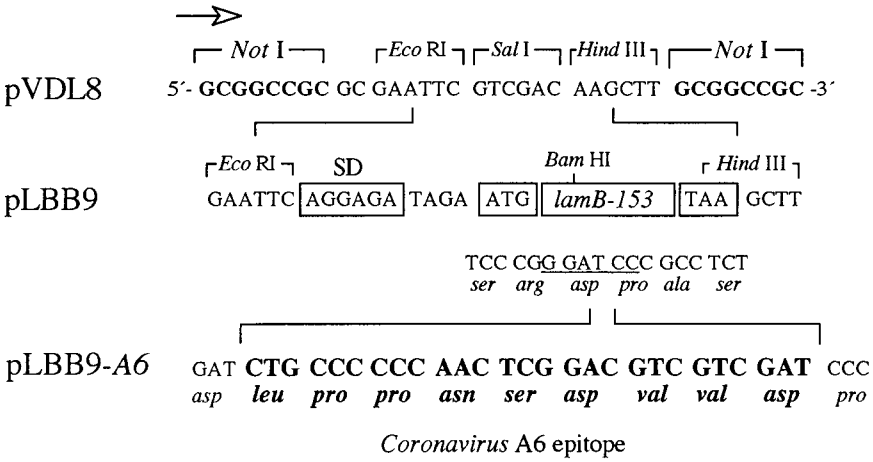

FIG. 3. Organization of the promoterless *lamB-A6* hybrid gene. The figure shows relevant sequences of the constructions used to generate the surface reporter gene. The polylinker region of low-copy-number vector pVDL8 is identical to that of p18Not (13). The orientation of the *lac* promoter present in the vector is indicated. A 1.4-kb *Eco*RI-*Hin*dIII restriction fragment spanning exactly the *lamB-153* sequence (same as wt *lamB* but with a novel *Bam*HI site engineered at structural codon 153) (2) was inserted in the corresponding sites of pVDL8, producing pLBB9. The ribosome-binding site (SD), the first structural codon (ATG), and the stop codon (TAA) are indicated at the extremes of the structural sequence. Insertion of a 27-bp linker at the *Bam*HI site of pLBB9 resulted in pLBB9-A6, which carries the TGEV A6 epitope and determines a hybrid protein in which this antigen is presented on the cell surface.

putida KT2442 as described in Materials and Methods. This ensured transcription of the *lamB* gene in a configuration such that all controlling elements were present under near native conditions and stoichiometry.

The results in Fig. 2 demonstrate that the *lamB* gene is transcribed, translated, and processed properly in *P. putida*, with no hint of degradation or accumulation of precursors, at least by judging the protein product detected in a Western blot (immunoblot) analysis. The size of LamB produced in *P. putida* in response to the presence in the medium of salicylate was identical to that of the mature protein in *E. coli*, with no uncleaved precursor (i.e., preprotein with the intact leader peptide), which was observed when the same protein was overproduced in *E. coli*. Furthermore, cell fractionation of *lamB*$^+$ *P. putida* cells showed that the LamB protein remained entirely bound to the outer membrane fraction (results not shown). However, the same strain was totally resistant to wt lambda phage regardless of the presence or absence of the LamB protein. These data indicated that entry of lambda DNA into recipient cells may require extra functions not present in *P. putida* (26). Alternatively, the protein may have been folded in a different conformation and/or could have been masked by other components of the cell envelope (i.e., lipopolysaccharide), thus rendering it unavailable to the phage. As explained below, these uncertainties were solved with the use of a hybrid LamB protein with a heterologous epitope inserted.

Cellular location and presentation of the antigenic site C of TGEV on the surface of *P. putida*. The desired versatility of the surface reporter system depicted in Fig. 1 requires the epitope used for immunological detection to be recognized by specific MAbs both in vivo and in Western blots, relatively independent of the conformation of the surrounding carrier protein. For this, we used the antigenic site C of the S glycoprotein of TGEV, hereafter designated the A6 antigen. This site encompasses the sequence Asp-Leu-Pro-Pro-Asn-Ser-Asp-Val-Val-Asp, whose core sequence, Pro-Pro-Asn-Ser-Asp-Val, strongly reacts with MAb 6A.A6 in various conformations (15). Genetic insertion of the extended antigen at the permissive position 153 of the amino acid sequence (Fig. 3) was made by ligating a synthetic linker to the single *Bam*HI site of plasmid pAJC264 (Table 1). This ligation gave rise to pCOR264, in which the resulting fused gene, *lamB-A6*, is transcribed through a *Ptac* promoter (Fig. 3). Prior to its expression in *P. putida*, we verified through Western blot analysis that the corresponding hybrid LamB-A6 was expressed in *E. coli* (results not shown). Furthermore, immunoblotting of intact cells and epifluorescence of the *lamB-A6*$^+$ strain treated with MAb 6A.A6 revealed that the heterologous epitope was accessible from the external medium (see Fig. 5A). *E. coli* cells expressing LamB-A6 were sensitive to mutant lambda phages λh^0 and λhh^* but not to wt lambda phage λh^+ (2, 3), thus indicating that the overall structure of the protein was conserved after insertion of the heterologous epitope.

Next, we engineered expression of LamB-A6 in *P. putida*. For this, we retrieved the *lamB-A6* sequence from pCOR264 by PCR with two primers which amplified a 1.4-kb DNA segment corresponding to a promoterless hybrid gene, which was finally flanked by *Not*I restriction sites in pLBB9-A6 (Table 1) and placed in front of the *m*-toluate-responsive *Pm* promoter of the TOL (toluene degradation) plasmid pWW0 (Fig. 1). The whole unit was then assembled in transposon mini-Tn*5 xylS*/*Pm*→*lamB-A6* (Table 1) and inserted into the chromosome of *P. putida* as described in Materials and Methods. Expression of the antigenic surface reporter in a number of inserted clones was examined. Western blot analysis of *P. putida* protein extracts (Fig. 4) showed that the TGEV antigen C-specific MAb 6A.A6 reacted with samples from cells induced with *m*-toluate but that no production of LamB-A6 was detected in the absence of the effectors of the native activator of *Pm*, the XylS protein (Fig. 4). Interestingly, the level of expression seemed to be somewhat dependent on the site of insertion. This phenomenon has been studied separately and seems to be due to changes in readthrough transcription of the *xylS* gene present in the transposon rather than to effects of differential superhelicity of the DNA sequences near the chromosomal location of the mobile unit (3a).

One of the strains giving a good signal in Western blot analysis, namely, *P. putida* KT2442-116A, was selected for further studies. Analysis of cell extracts derived from this strain confirmed that, similarly to the wt LamB protein (see above), the hybrid produced in *P. putida* was the same size as the one produced in *E. coli* and that LamB-A6 partitioned with the

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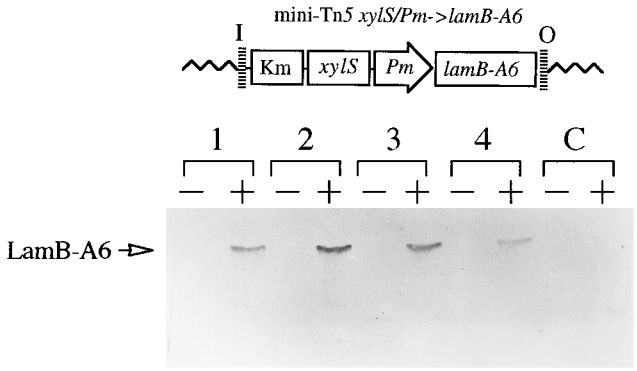

FIG. 4. Expression of LamB-A6 in *P. putida*. Different exconjugants of the mating S17-1λ*pir* (pTCOR12) × *P. putida* KT2442 (labelled 1 to 4) carrying insertions of mini-Tn*5* *xylS*/*Pm*→*lamB-A6* (Fig. 1) at various chromosomal positions were grown at 30°C in LB to an optical density at 600 nm of 0.5 and induced for 5 h with 2 mM *m*-toluate (lane +) or left uninduced (lane −). Samples of each exconjugant were run in a denaturing polyacrylamide gel, blotted on a membrane, probed with a 1:5,000 dilution of the MAb 6A.A6, and further developed with goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase, as described in Materials and Methods. Lane C is a control consisting of *P. putida* KT2442 with no insertions. The position of the hybrid LamB-A6 protein is indicated to the left of the figure.

outer membrane protein fraction of lysed cells (data not shown). Finally, we examined the external availability of the reporter epitope for reaction with MAb 6A.A6 through standard epifluorescence microscopy. The results in Fig. 5B show unequivocally that expression of the reporter epitope can be monitored through its exposure on the cell envelope in a fashion which permits its recognition by a cognate antibody. Taken together, these experiments show that the LamB protein and its derivative LamB-A6, carrying the TGEV epitope, are transcribed, translated, folded, processed, and exported to the outer membrane of *P. putida* similarly to the same processes in *E. coli*. Furthermore, the heterologous antigen is placed on the cell envelope and properly presented for reaction with a specific MAb. Therefore, the hybrid LamB-A6 protein seems to perform well as a marker system for nondisruptive detection of antigen-producing single cells by using immunological procedures. Although we have not made a systematic quantitative study of promoter activity versus reporter readout, strains carrying an equivalent *lacZ* fusion to *Pm* in mini-Tn*5* *xylS*/*Pm* (Table 1) gave levels of β-galactosidase ranging from 50–100 Miller units (uninduced) to 3,000–6,000 units (culture amended with *m*-toluate). Such a variation could be correlated with expression of the hybrid LamB protein in Western blots (data not shown). Visual inspection of fluorescence of cells under the microscope had, in principle, a lesser quantitative meaning while being perfectly adequate to visualize the on and off promoter switch under various growth conditions.

Monitoring activity of the *Pm* promoter of the TOL plasmid within a bacterial community. The sections above have dealt with the use of the hybrid LamB-A6 protein in monocultures and under the defined conditions used in our laboratory. The next question to address was the utility of the system to track promoter activity in mixed communities, in which the cells under study might be just a very minor component of the microbial population. We examined this issue by inoculating a consortium of various bacterial species (*Bordetella bronchiseptica*, *Salmonella typhimurium*, *E. coli*, *P. putida*, and *Rhizobium tropici*) with *P. putida* KT2442-116A in a medium containing *m*-toluate. As shown in Fig. 5C and D, the subpopulation of cells containing a transcriptionally active *Pm* promoter could be perfectly distinguished from the rest of the consortium. In a further step, we inoculated the same *Pseudomonas* strain in the activated sludge of a treatment plant for terminal effluents of a petroleum refinery amended with 2 mM *m*-toluate. In this last case, the composition of the native microbial population was complex and largely undefined and contained a considerable component of particulate and solid materials. Figure 5E and F indicates that the *Pm* promoter switching on could be assessed equally well in individual cells. Furthermore, inspection of the samples in the epifluorescence microscope indicated also that the *P. putida* KT2442-116A strain under examination had a tendency to form clusters and microcolonies within the milieu of the activated sludge rather than existing as isolated bacteria.

Nondisruptive monitoring of promoter switching on and switching off with surface reporters. Unlike other procedures used so far to monitor the activity of bacterial promoters, the indicator system described in this work relies on a physical property of the reporter product (its recognition by a cognate antibody) instead of an enzymatic or optical trait. The extremely rare amino acid sequence of the TGEV epitope used guarantees its specific recognition by its matching MAbs, even when the cells under study are a very minor component of a complex bacterial population. Although we have used the LamB-A6 protein in this work to examine the activity of the *m*-toluate-responsive catabolic promoter *Pm* of the TOL plasmid for biodegradation of toluene in an activated sludge, its utilization can be generally considered also in cases when transcription is to be assessed in environments where the cells under scrutiny must be studied in situ.

Only two general limitations to the use of the reporter system described in this work can be anticipated, namely, inefficient export of the hybrid to the cell surface and occlusion of the LamB-carried epitope by other components of the cell envelope. As mentioned above, we have not found any bottleneck in the expression and secretion of the LamB protein or its hybrid derivative within the range of transcriptional activities afforded by the *Pm* promoter in monocopy gene dosage in *P. putida*, which is one of the strongest known (19). Although functionality of signal sequences for protein transport across bacterial membranes seems to be conserved throughout prokaryotes (18, 25, 37, 38), this step may become a limitation in some strains (10). A more serious concern is the accessibility of the antigen to antibodies if the strain under study produces a bulky lipopolysaccharide. For instance, expression of a hybrid PhoE-ColA protein was hardly detected with anti-colicin A MAb in wt *P. putida* WCS358 cells but was readily observed in mutants lacking part of the O antigen (40). In contrast, our data showed that the exposed loop of LamB chosen to insert the coronavirus antigen in the hybrid protein was protruding enough to be externally accessible to antibodies in *P. putida* KT2442 under the nondisruptive conditions used. Besides *P. putida* KT2442, we have successfully used the same system to label root-colonizing *P. fluorescens* (not shown), and we anticipate that surface reporters of this type should have a very general applicability in other gram-negative bacteria.

An obvious development of the LamB-based surface reporter system is its coupling to promoter-probing schemes for identification of genes responsive to environmental stimuli that cannot be easily reproduced in the assay tube. In that context, we have recently constructed a specialized mini-Tn*5* transposon for random generation of transcriptional fusions to a promoterless *lamB-A6* gene. The use of such a genetic tool to investigate signals sensed in situ by *Pseudomonas* strains thriving in ecosystems polluted with aromatic chemicals is being studied.

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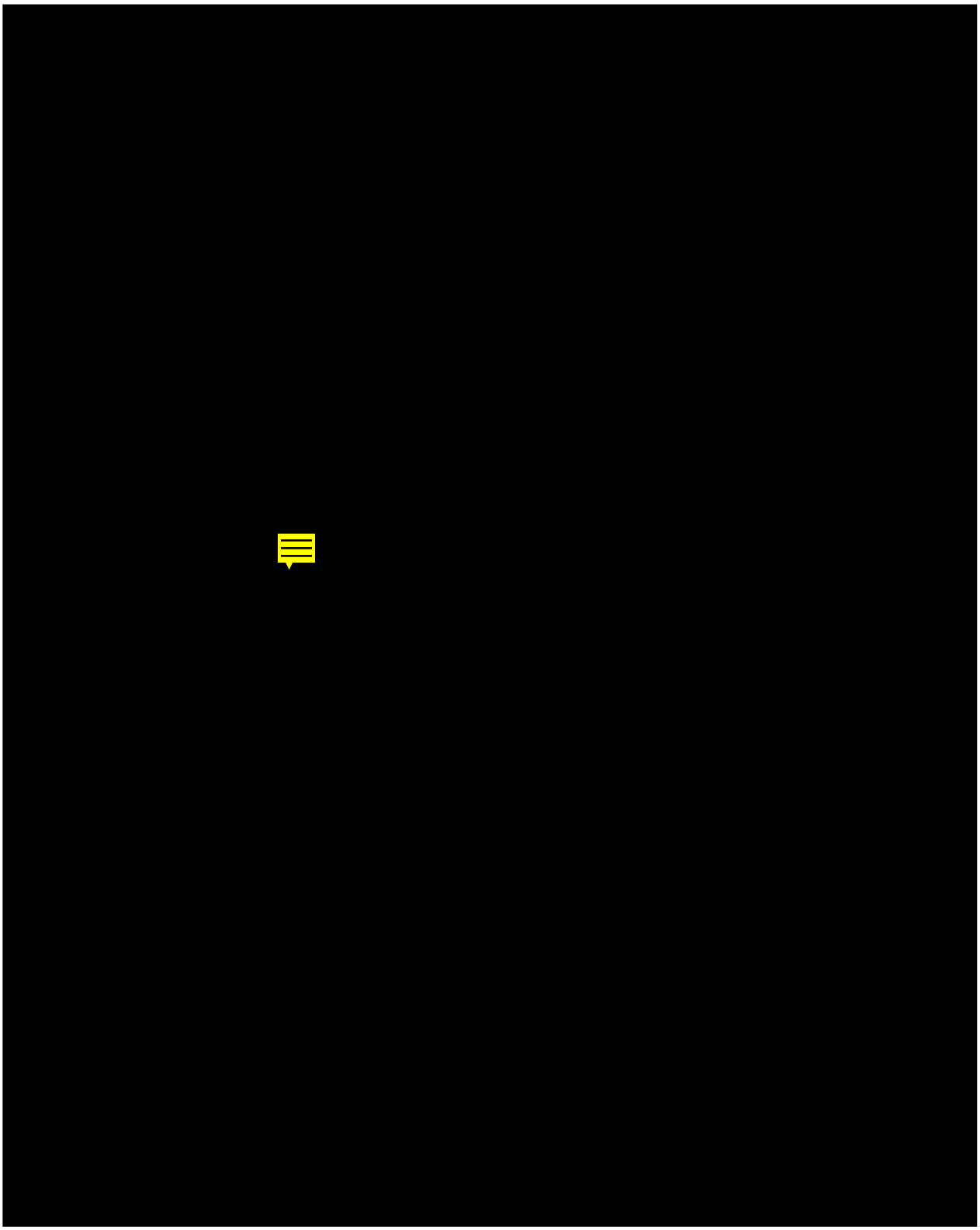

FIG. 5. Epifluorescence microscopy of cells presenting the TGEV A6 epitope to the external medium. The different samples were heat fixed and treated with the anti-A6 MAbs, incubated with fluorescein-labelled goat anti-mouse immunoglobulin G, and then examined in a fluorescence microscope. (A) Control, *lamB-A6*$^+$ *E. coli* POP6510-126A cells grown in LB and induced with *m*-toluate. (B) *lamB-A6*$^+$ *P. putida* KT2442-116A cells grown in LB and induced with *m*-toluate. Uninduced cells gave a totally dark field (not shown). (C) DAPI staining of a bacterial consortium including *P. putida*, *E. coli*, *B. bronchiseptica*, *R. tropici*, and *S. typhimurium* and *m*-toluate-induced *lamB-A6*$^+$ *P. putida* KT2442-116A. (D) Same sample and field as in panel C, but the image was filtered for fluorescein isothiocyanate fluorescence. (E) Phase-contrast micrograph of a sample of activated sludge from an oil refinery treatment plant with *m*-toluate-induced *lamB-A6*$^+$ *P. putida* KT2442-116A cells. (F) Same sample and field as in panel E, but the image was filtered for fluorescein isothiocyanate fluorescence. Note that some components of the particulate material of the activated sludge may display intrinsic fluorescence at a different wavelength.

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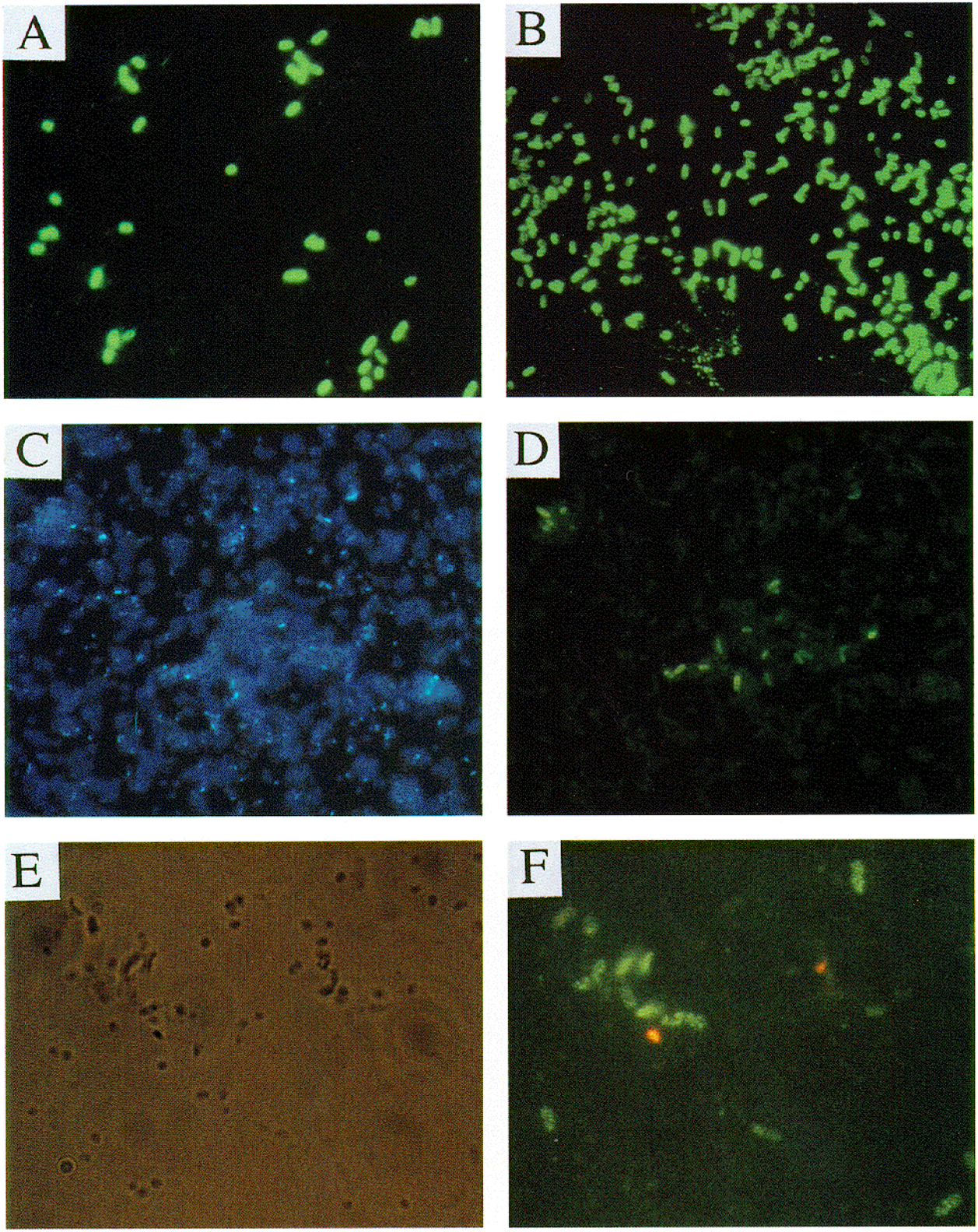
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