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Construction of *phoE-caa*, a Novel PCR- and Immunologically Detectable Marker Gene for *Pseudomonas putida*

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In this paper we describe the construction and use in *Pseudomonas putida* WCS358 of *phoE-caa*, a novel hybrid marker gene, which allows monitoring both at the protein level by immunological methods and at the DNA level by PCR. The marker is based on the *Escherichia coli* outer membrane protein gene *phoE* and 75 bp of *E. coli caa*, which encode a nonbacteriocinic fragment of colicin A. This fragment contains an epitope which is recognized by monoclonal antibody (MAb) 1C11. As the epitope is contained in one of the cell surface-exposed loops of PhoE, whole cells of bacteria expressing the protein can be detected by using the MAb. The marker gene contains only *E. coli* sequences not coding for toxins and therefore can be considered environmentally safe. The hybrid PhoE-ColA protein was expressed in *E. coli* under conditions of phosphate starvation, and single cells could be detected by immunofluorescence microscopy with MAb 1C11. Using a wide-host-range vector the *phoE-caa* gene was introduced into *P. putida* WCS358. The gene appeared to be expressed under phosphate limitation in this species, and the gene product was present in the membrane fraction and reacted with MAb 1C11. The hybrid PhoE-ColA protein could be detected on whole cells of WCS358 mutant strains lacking (part of) the O-antigen of the lipopolysaccharide but not on wild-type WCS358 cells, unless these cells had previously been washed with 10 mM EDTA. In addition to immunodetection, the *phoE-caa* marker gene could be specifically detected by PCR with one primer directed to a part of the *phoE* sequence and a second primer that annealed to the *caa* insert.

Recombinant DNA techniques have largely facilitated the construction of specialized bacterial strains for agricultural and industrial purposes. Of the bacterial species which have potential as enhancers of plant growth, some fluorescent pseudomonads show particular promise (24). This plant growth promotion requires efficient colonization of the plant root system. One obvious possibility to further improve the performance of selected strains is the use of root colonization genes (24). Prior to application in the environment of bacteria equipped with such genes, however, insight has to be obtained in the spreading of the bacteria and of their DNA.

In recent years an increasing number of molecular techniques have been applied to monitoring bacteria (28, 30, 37). The most rapid sensitive methods for screening large numbers of samples probably are monoclonal antibody (MAb)-based techniques (31) and PCR analysis. For in situ studies of recombinant bacteria, immunological methods are the most appropriate, since they allow single cell microscopic detection without disrupting the microenvironment (22, 32).

For our studies with recombinant root-colonizing *Pseudomonas* strains, we needed a marker that would allow very sensitive detection in large numbers of soil samples as well as samples from groundwater. Furthermore, to be able to study the root colonization process in detail, the marker should allow detection of single bacterial cells on root systems. We therefore constructed a novel marker gene based on the *Escherichia coli phoE* and *caa* genes, which can be detected by MAbs as well as by PCR.

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The *E. coli phoE* gene is regulated as part of the *pho* regulon, which is induced under conditions of phosphate starvation (27). The gene encodes the 33-kDa PhoE protein, which is incorporated in the outer membrane as a trimer and functions as an anion-selective pore (6). A model for the folding of the PhoE monomer of 16 membrane-spanning domains connected by periplasmic and outer cell surface-exposed loops (41) was recently confirmed by the resolution of the crystal structure of the protein (9). The protein has been used as a carrier for cell surface exposure of foreign epitopes in *E. coli* (2).

Under conditions of phosphate starvation, *Pseudomonas aeruginosa* expresses the OprP outer membrane pore protein, a phosphate uptake system which has a function similar to that of *E. coli* PhoE (17). The regulatory circuits for the expression of the *phoE* and *oprP* genes are well conserved. In *E. coli* the bifunctional repressor-activator PhoR protein is converted to its activator form under conditions of phosphate starvation and activates PhoB, which in turn activates transcription of the structural genes of the *pho* regulon. This gene activation involves interaction of activated PhoB with regulatory sequences, designated *pho* boxes (43), which precede the structural genes. The *P. aeruginosa oprP* gene is preceded by a *pho* box, which is also functional in *E. coli* (33). Furthermore, *P. aeruginosa phoB*- and *phoR*-like regulatory genes can complement *phoB* and *phoR* mutations in *E. coli*, respectively (13). Conversely, expression of the *phoE* gene in *P. aeruginosa* PAO286 results in the incorporation of a functional PhoE protein in the outer membrane (42). Thus, we assumed that the *E. coli phoE* gene would be effectively expressed in *Pseudomonas putida*, without conferring a major selective advantage to the latter bacterium. We therefore decided to use the PhoE protein as a carrier for a specific epitope, in order to mark *P. putida*. For this purpose we chose for the *E. coli* colicin

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli K-12		
KMBL1164	del *lac-pro*	P. Van der Putte
CE1248	*ompR phoE phoR*	20
Pseudomonas sp.		
WCS358	*P. putida* wild type	14
LWP358-5c	O-antigen mutant of WCS358 (truncated O-antigen)	12
LWP358-43b	O-antigen mutant of WCS358 (O-antigen absent)	12
WCS007	*P. putida* wild type	11
WCS134	*Pseudomonas* sp. wild type	11
WCS307	*P. fluorescens* wild type	11
WCS315	*Pseudomonas* sp. wild type	11
WCS379	*Pseudomonas* sp. wild type	11
M114	*Pseudomonas* sp. wild type	25
Plasmids		
pMR05	pACYC184 derivative carrying a *phoE* gene in which an *Nru*I site was generated in the region encoding Arg-158	4
pMP2424	pMR05 containing the first *caa* fragment in correct orientation and the second fragment in reverse orientation (Fig. 1)	This study
pMP2425	pMR05 containing total *caa* insert in correct orientation	This study
pMP92	RK2-derived IncP group wide-host-range cloning vector	35
pMP2427	pMP92 containing hybrid *phoE-caa* gene of pMP2425	This study

A epitope, which is recognized by MAb 1C11 (15). Detection of the hybrid PhoE protein was investigated in wild-type and O-antigen mutant strains of *P. putida*. In addition to MAb detection, we investigated detection of the marker gene by PCR, using primers directed against the *phoE* and *caa* sequences.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids with their relevant characteristics are listed in Table 1. *E. coli* strains were grown overnight on solidified or in liquid LC (34) or phosphate-limited medium (17), supplemented, if necessary, with chloramphenicol or tetracycline (both at 20 μg/ml), at 37°C. *P. putida* strains were grown on solidified or in liquid King's medium B (19) without added Mg_2SO_4 or in phosphate-limited medium, supplemented with 80 μg of tetracycline per ml, if required.

Soil microflora. An air-dried sample of 10 g of loamy sand potato-cropping soil (26) was hydrated overnight with 2 ml of tap water, at 4°C. The soil was extracted with 18 ml of phosphate-buffered saline (PBS [10 mM NaH_2PO-Na_2HPO_4, 9% NaCl] [pH 7.4]) by vigorous mixing for 10 min at room temperature with a whirl mixer. After centrifugation for 10 min at 75 × *g*, 50-μl aliquots of serial 10-fold dilutions of the supernatant were plated on LC and phosphate-limited media and incubated at 28°C overnight and for 2 days, respectively.

DNA techniques. Oligonucleotides were synthesized on a Biosearch 8600 DNA synthesizer and were purified by high-performance liquid chromatography. Plasmid and chromosomal DNA was isolated after alkaline lysis (7, 29) by CsCl-ethidium bromide isopycnic centrifugation. DNA concentrations were determined spectrophotometrically (29). Restriction enzymes and T4 DNA ligase were purchased from Pharmacia (Woerden, The Netherlands). Cloning, preparation of transformation-competent cells of *E. coli*, and transformations were carried out according to standard procedures (29). Inserts in the *Nru*I site of the *phoE* gene (see Fig. 1) were sequenced with Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) according to the manufacturer's protocol, with 1 to 5 μg of double-stranded DNA from a Qiagen (Diagen GmbH, Düsseldorf, Germany) plasmid minipreparation as the template and 1 pmol of primer 85.29 (5'-ACG TATCGGAACACCGAC-3'), which anneals 59 bp upstream from the *Nru*I site in the *phoE* gene. Transfer of plasmids to *P. putida* was performed by electroporation with a gene pulser (Bio-Rad Laboratories, Richmond, Calif.). Cells were prepared and electroporated by using the manufacturer's protocol for *E. coli*.

Isolation and characterization of membrane fractions. For analysis of native or mutant PhoE proteins in *E. coli*, cells of strain CE1248 were grown in LC, since the *phoR* mutation in this strain abolishes the need for phosphate limitation to express the *phoE* gene. *P. putida* strains to be analyzed were grown in phosphate-limited medium. Membrane fractions were obtained by ultrasonic lysis and differential centrifugation. *E. coli* membrane preparations were analyzed on sodium dodecyl sulfate (SDS)–11% polyacrylamide gels according to the method of Lugtenberg et al. (23). *P. putida* membrane preparations were subjected to Sarkosyl extraction (11) and analyzed on SDS–11% polyacrylamide gels according to the method of Laemmli (21). Molecular mass marker positions are those from the calibration proteins lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), glutamate dehydrogenase (55 kDa), bovine serum albumin (BSA; 66 kDa), and phosphorylase *b* (97 kDa).

Western blotting and immunodetection. Proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) were transferred to nitrocellulose sheets (BA85; Schleicher & Schuell, Dassel, Germany) by electroblotting (38). The sheets were blocked with 2% (wt/vol) defatted milk powder in Tween buffer (TB; 0.1% Tween in PBS) for 1 h and incubated with a 2,000-fold-diluted solution of MAb 1C11 ascites fluid (15) in

TB for 1 h. After being washed in three changes of TB for 30 min, blots were incubated with 2,000-fold-diluted alkaline phosphatase- or horseradish peroxidase-conjugated rabbit anti-mouse serum (Sigma Chemical Co., St. Louis, Mo.) in TB for 1 h, washed again in TB for 30 min, and subsequently developed. Nitrotetrazolium blue and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates for alkaline phosphatase (11). Peroxidase was detected with dioctyl sulfosuccinaat (DONS) and tetramethylbenzide (TMB). For this purpose, 40 mg of DONS and 12 mg of TMB were dissolved in 5 ml of ethanol of 65°C, cooled to room temperature, and mixed with 15 ml of phosphate-citrate buffer (4.85 ml of 100 mM citric acid plus 5.15 ml of 200 mM Na_2HPO_4 in 100 ml [pH 5.0]). The reaction was started by the addition of 10 μl of 30% H_2O_2 and stopped by extensive washing with tap water.

Colony blotting and immunodetection. Bacterial colonies from fresh plates containing phosphate-limited medium were streaked or blotted onto nitrocellulose (BA85) or prewetted immobilon membranes (polyvinylidene fluoride; Bio-Rad, Venendaal, The Netherlands). After the removal of excess bacteria by washing with demineralized water, immunodetection was performed as described for Western blots (immunoblots) with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) as the second antibody and DONS and TMB as the substrates.

Immunofluorescence microscopy. *E. coli* CE1248.pMP2425 and CE1248.pMR05 were grown overnight in LC medium supplemented with 20 μg of chloramphenicol per ml. Cells from 1.0 ml of culture were harvested by centrifugation and resuspended in 1.0 ml PBS–10 mM glycine, and droplets of 10 and 20 μl were spotted on silane-coated glass slides and dried at 37°C. The slides were incubated in blocking buffer (BB; PBS, 10 mM glycine, 0.1% fish gelatin, 0.8% BSA) for 20 min at room temperature, rinsed with PBS–10 mM glycine, air dried, and incubated for 1 h at 37°C with MAb 1C11 ascites fluid diluted 250-fold in BB. After the slides were carefully washed three times with PBS–10 mM glycine, they were incubated with rabbit anti-mouse IgG–fluorescein isothiocyanate (FITC) (Nordic Immunochemical Laboratories, Tilburg, The Netherlands) (diluted 40-fold in BB) for 40 min at 37°C, washed with PBS–10 mM glycine, incubated with goat anti-rabbit immunoglobulin G–FITC (Nordic) (diluted 200-fold in BB) for 40 min at 37°C, washed, air dried, and embedded in Cityfluor (Agar Scientific Ltd., Cambridge, United Kingdom). The slides were inspected with a Laborlux D epifluorescence microscope (Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) with an I2 filter block (BP450-490, RKP50, and LP515) and equipped with a Wild MPS51S photo camera. Photographs were taken with Kodak Ektachrome 400 ASA films and commercially developed.

PCR detection. Isolated plasmid DNA (1 ng) or chromosomal DNA (100 ng) was analyzed with 25 pmol of either primers pr28 (5′-GCAGAGAGTGATACTGC-3′) and pr8 (5′-AATATCATCATTATTAATATTCAA) or primers pr28 and pr3 (5′-GATCGGAAAAGGTGATGGTACCGGCTGG AGCTCAGAACGTG-3′) with 2.5 U of Replitherm thermostable DNA polymerase (Epicenter Technologies, Madison, Wis.) in a 100-μl reaction volume overlaid with 100 μl of mineral oil, by using a PREM thermal cycler (Biozym, Landgraaf, The Netherlands) programmed for 30 cycles of 1 min at 95°C, 2 min at 37°C, and 2 min at 72°C. Primer pr3 is based on the MAb 1C11 epitope-encoding part of *caa* (16). Primers pr28 and pr8 are based on the *E. coli phoE* sequence (40). Primer pr8 has been used previously as an enterobacterial probe (36). The positions of the primers on the hybrid *phoE-caa* gene are depicted in Fig. 7. In later experiments, primer pr3 was replaced by its derivative pr7 (5′-CGTCGGAAAAGGTGAT GGTACCGGCTGGAG-3′), and prim-er pr8 was replaced by its derivative pr1 (5′-AATATTCAATTTGTTATCGCTAT CC-3′), allowing an annealing temperature of 50°C in an otherwise unmodified PCR program. Each reaction mixture (10 μl) was analyzed on a 1% agarose gel and visualized by ethidium bromide staining. *Msp*I-digested plasmid pBR322 DNA (0.5 μg; New England Biolabs, Beverly, Mass.) was used as marker.

RESULTS

Construction of *phoE-caa* hybrid gene. To study the potential of the *E. coli* PhoE outer membrane protein as a carrier for cell surface-exposed epitopes to mark *P. putida*, a *phoE-caa* hybrid gene was constructed. For this purpose, plasmid pMR05, which carries a *phoE* gene with an *Nru*I restriction site in the sequence coding for Asn-157 to Asp-159, which forms part of the fourth cell surface-exposed loop of the PhoE protein, was used. Amino acid residues encoded by DNA inserted at this *Nru*I site will be exposed at the cell surface (2). A 75-bp fragment of the *E. coli caa* gene, which encodes a region carrying an epitope for MAb 1C11, was selected. This fragment, encoding Gly-8 to His-30 (16), was generated by sequential insertion of two synthetic DNA fragments (Fig. 1). Plasmid pMR05 was linearized with *Nru*I and ligated to fragment 1 (Fig. 1A). To eliminate self-annealed plasmid, the ligation mixture was digested with *Nru*I prior to transformation of *E. coli* KMBL1164. Plasmid DNA from chloramphenicol-resistant transformants was isolated and digested with *Sal*I, since insertion of fragment 1 in the correct orientation would create a *Sal*I site 3′ and a *Bam*HI site 5′ to the insert. One of the plasmids containing the fragment in this orientation was linearized with *Bam*HI, ligated to synthetic *caa* fragment 2 (Fig. 1B), and used to transform *E. coli* KMBL1164 cells. Proper insertion of fragment 2 would result in a *Bam*HI site at the 3′ end of fragment 2, at 34 bp from the *Sal*I site created by the insertion of fragment 1. Incorrect insertion would generate a *Bam*HI site at the 5′ end of fragment 2, at 75 bp from the *Sal*I site. Isolated plasmid DNA of chloramphenicol-resistant transformants was digested with *Bam*HI and *Sal*I and analyzed on a nondenaturing 20% polyacrylamide gel. Three plasmids containing a 34-bp *Bam*HI-*Sal*I fragment and one plasmid containing a 75-bp *Bam*HI-*Sal*I fragment, designated pMP2424, were sequenced. pMP2424 contained fragment 2 in inverted orientation, as was expected from the restriction analysis. One of the three plasmids mentioned previously, which all contained the total 75-bp *caa* sequence properly inserted in *phoE*, was designated pMP2425 (Fig. 1C) and used for subsequent expression studies and cloning.

Immunodetection of PhoE-ColA protein in *E. coli*. Plasmid pMP2425 was electroporated into cells of *E. coli* CE1248. Since this strain lacks the *phoR* repressor function as well as the *phoE* structural gene, the cells constitutively express the *phoE-caa* gene of plasmid pMP2425. Membranes of strains CE1248, CE1248.pMP2425, and CE1248.pMR05 were isolated and analyzed by SDS-PAGE and Western blotting with MAb 1C11 (15). Figure 2A shows that the mutant PhoE protein encoded by plasmid pMP2425 is indeed present in the membrane preparation, indicating that the inserted 25 amino acid residues do not interfere with membrane insertion of the PhoE-ColA hybrid protein. The hybrid PhoE-ColA protein shows a reduced migration in the gel compared with the wild-type PhoE protein of CE1248 pMR05, because of the 25 extra amino acid residues (Fig. 2A). The level of expression of the mutant protein is reduced in comparison with that of the

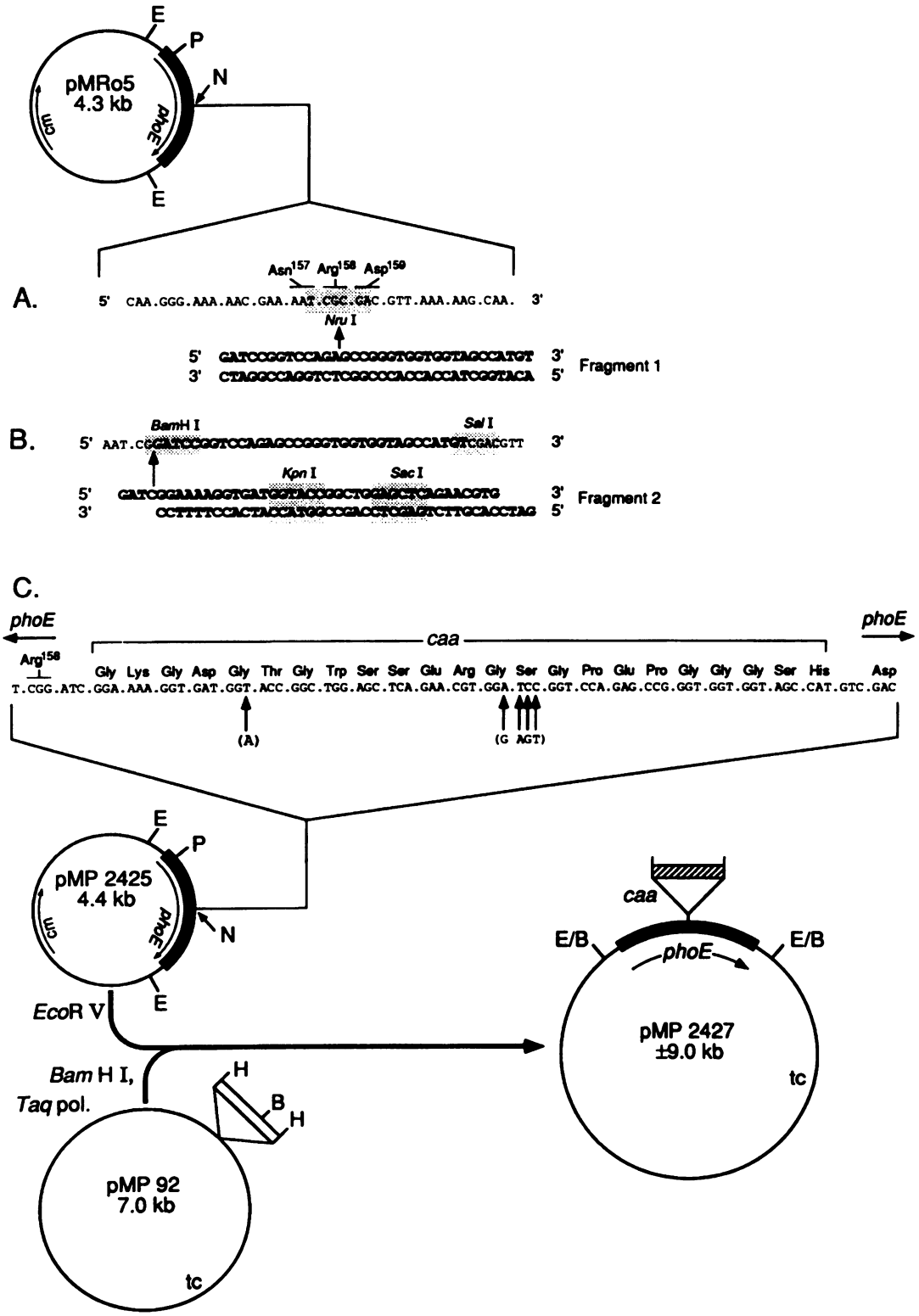

FIG. 1. Cloning strategy for insertion of *caa* fragment which codes for a peptide epitope recognized by MAb 1C11 into *phoE* and construction of a wide-host-range vector carrying the hybrid *phoE-caa* gene. (A) Insertion of first part of *caa* fragment into *phoE*; (B) insertion of second part of *caa* into *phoE*; (C) Total *caa* insert in *phoE* and construction of wide-host-range vector carrying the *phoE-caa* gene. Nucleotides of the original *caa* gene which were replaced to create restriction sites without altering the coding sequence are depicted in parentheses. B, *Bam*HI; E, *Eco*RV; P, *Pst*I; *Taq* pol., *Taq* DNA polymerase; cm, chloramphenicol; tc, tetracycline.

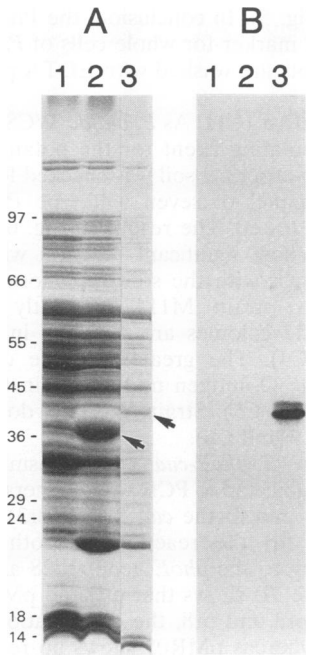

FIG. 2. Analysis of membrane proteins of *E. coli* strains with mutant *phoE* genes. (A) Fast green-stained protein profiles of membrane fractions of strains CE1248 (lane 1), CE1248.pMR05 (lane 2), and CE1248.pMP2425 (lane 3); wild-type (lane 2) and immunoreactive (lane 3) PhoE proteins are indicated by arrows. The positions of molecular weight markers (in kilodaltons) are indicated on the left. (B) Western blot of the samples described in A, incubated with MAb 1C11.

wild-type PhoE. A low level of expression has been observed previously with other altered PhoE proteins in *E. coli* CE1248 (1). The Western blot (Fig. 2B) clearly shows that only the hybrid PhoE-ColA protein encoded by pMP2425, and not the PhoE wild-type protein, is recognized by MAb 1C11.

Single cell detection of *E. coli* CE1248 pMP2425 by immunofluorescence microscopy. To test whether the ColA epitope was exposed at the outer cell surface of *E. coli* CE1248 cells and whether its expression was sufficient for single cell detection, CE1248.pMP2425 and CE1248.pMR05 cells were adhered mildly to silicon-coated glass slides. To avoid damage to the cells a fixation procedure was avoided. The slides were treated with MAb 1C11, followed by double immunofluorescence labelling with rabbit anti-mouse IgG–FITC and goat anti-rabbit IgG–FITC. Immunofluorescence microscopy revealed that single CE1248.pMP2425 cells were clearly stained (Fig. 3), whereas the CE1248.pMR05 wild-type PhoE control cells showed no signal.

Expression of *phoE-caa* in *P. putida* WCS358. The *phoE-caa* gene, including its native promoter and upstream controlling sequences (*pho* boxes), was excised from plasmid pMP2425 as an *Eco*RV fragment and ligated to *Bam*HI-digested wide-host-range plasmid pMP92 (35), after the sticky ends of the linearized vector were filled with Replitherm thermostable DNA polymerase (Fig. 1C). After electroporation of *E. coli* KMBL1164, DNA of tetracycline-resistance transformants was isolated and digested with *Pst*I to determine the orientation of the insert. One plasmid, designated as pMP2427, which carries the *phoE-caa* gene in the orientation depicted in Fig. 1C, was transferred to *P. putida* WCS358 by electroporation.

E. coli KMBL1164.pMP2427 and *P. putida* WCS358.pMP

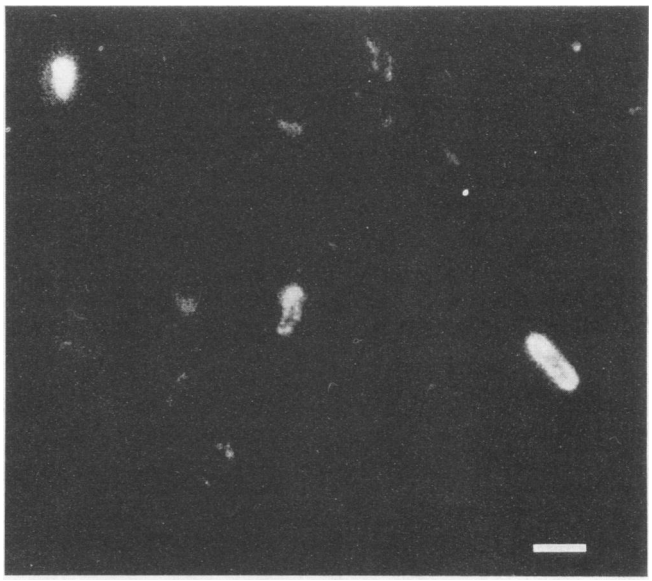

FIG. 3. Whole-cell immunofluorescence detection of *E. coli* CE1248.pMP2425 expressing the hybrid PhoE-ColA protein. Bar, 1 μm.

2427 were grown on phosphate-limited medium, and expression and detectability of the PhoE-ColA hybrid protein were assayed with MAb 1C11 by colony blotting on nitrocellulose sheets. KMBL1164.pMR05 (wild-type *phoE*) and KMBL 1164.pMP2425 served as negative and positive controls, respectively. KMBL1164.pMP2427 showed a signal comparable to that of KMBL1164.pMP2425, indicating that in *E. coli* KMBL1164 the *phoE-caa* gene on plasmid pMP2427 is properly expressed under phosphate limitation. *P. putida* WCS358.pMP2427, however, did not show more signal than the negative control in the colony blot (data not shown). This finding could mean that either the *phoE-caa* gene of pMP2427 is not expressed under phosphate limitation in *P. putida* WCS358 or the gene is expressed but that the ColA epitope is not accessible for MAb 1C11 on whole cells of this bacterium.

To discriminate between these possibilities, Sarkosyl extracts of membrane preparations of *P. putida* WCS358 and WCS 358.pMP2427 grown in the absence and presence of phosphate were analyzed by SDS-PAGE and Western blotting with MAb 1C11, with the total membrane fractions of CE1248.pMP2425 and of CE1248.pMR05 as controls. Figure 4 shows that the membrane fraction of WCS358.pMP2427 grown under phosphate starvation contains a PhoE-ColA protein which reacts with MAb 1C11 (lanes A3 and B3) and which has the same size as PhoE-ColA in CE1248.pMP2425 (lanes A5 and B5). This protein is absent when WCS358 lacks plasmid pMP2427 (lane B1) or when WCS358.pMP2427 is grown in the presence of phosphate (lane B4). Thus, the *phoE-caa* gene is expressed under phosphate limitation in WCS358.pMP2427, and the PhoE-ColA protein most likely is incorporated in the outer membrane. The lack of detection of the marker on whole cells therefore must be caused by inaccessibility of the epitope for the MAb at the cell surface.

Detection of PhoE-ColA in *P. putida* WCS358 O-antigen mutant strains. Plasmid pMP2427 was electroporated into WCS358 O-antigen mutants LWP358-5c (short O-antigen) and LWP358-43b (no O-antigen) (12). The resulting strains were grown on phosphate-limited medium and tested for detectabil-

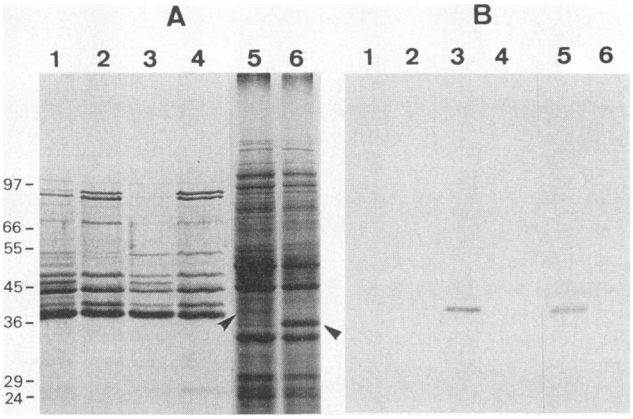

FIG. 4. Analysis of PhoE-ColA expression in *P. putida* WCS358. (A) Coomassie brilliant blue-stained outer membrane protein profiles of *P. putida* WCS358 (lanes 1 and 2) and WCS358.pMP2427 (lanes 3 and 4) grown in phosphate-limited (lanes 1 and 3) and phosphate-rich (lanes 2 and 4) media. Total membrane preparations of *E. coli* CE1248.pMP2425 (lane 5) and CE1248.pMR05 (lane 6) were used as controls. Arrows indicate hybrid (lane 5) and wild-type (lane 6) PhoE proteins. The positions of molecular weight markers (in kilodaltons) are indicated on the left. (B) Western blot of the samples used for panel A, after incubation with MAb 1C11. Sample volumes were adjusted in order to assay approximately equal amounts of protein.

ity with MAb 1C11 by immunoblotting on nitrocellulose. The O-antigen mutant strains carrying plasmid pMP2427 were indeed stained, whereas WCS358.pMP2427 showed only a faint reaction (Fig. 5). This indicates that on whole cells the O-antigenic side chain of the LPS of strain WCS358 is involved in shielding of the PhoE-ColA hybrid protein from antibody molecules.

Improving immunodetection of whole cells of *P. putida* WCS358 expressing hybrid PhoE-ColA protein. Cells of strains WCS358, WCS358.pMP2427, LWP358-5c, and LWP358-5c.pMP2427 grown on phosphate-limited medium were blotted onto nitrocellulose and immobilon membranes, which subsequently were washed three times for 20 min with demineralized water or with 50 mM Tris (pH 7.5), supplemented with either 10 mM EDTA, 2% or 5% Triton X-100, 2% or 5% SDS, or 2% SDS plus 2% β-mercaptoethanol prior to immunodetection. Most of the treatments removed the cells from the blots, as judged from the lack of reaction of the positive control LWP358-5c.pMP2427. Washing with Tris–10 mM EDTA, however, greatly improved the detectability of WCS

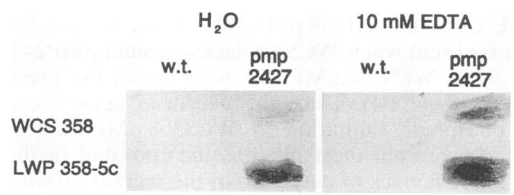

FIG. 5. Immunodetection of PhoE-ColA protein on whole cells of *P. putida* WCS358 wild type (w.t.) and O-antigen mutant strain LWP358-5c streaked on Immobilon membranes. Results with O-antigen mutant strain LWP358-43b were similar to those with LWP358-5c. Blots were washed three times with demineralized water or with 10 mM EDTA for 10 min prior to immunodetection with MAb 1C11.

358.pMP2427 (Fig. 5). In conclusion, the PhoE-ColA protein can be used as a marker for whole cells of *P. putida* WCS358 provided that blots are washed with EDTA prior to immunodetection.

Specificity of MAb 1C11. As *P. putida* WCS 358 will be used as a growth-stimulating agent for the potato, the microflora present in potato-cropping soil was checked for reactivity with MAb 1C11. A panel of seven wild-type *Pseudomonas* soil isolates was also tested. The results in Fig. 6 show that MAb 1C11 does not show significant reaction with any of these strains (Fig. 6A) or with the soil microflora (Fig. 6B). Only *Pseudomonas* sp. strain M114 is faintly stained. Strain WCS358.pMP2427 colonies are detected in the colony blot (Fig. 6C1 and C4). The greatest degree of reactivity was observed with the O-antigen mutant strain LWP358-5c.pMP 2427 (Fig. 6C2 and C5). Strain WCS358 does not show any reaction (Fig. 6C3 and C6).

PCR detection of *phoE-caa* gene. Plasmids pMR05 and pMP2425 were subjected to PCR with primers annealing to the *phoE* gene (pr8) and to the *caa* insert (pr3) (Fig. 7A). As a positive control for the reaction on both templates, two primers annealing to the *phoE* gene (pr28 and pr8; Fig. 7A) were used. Figure 7B shows that plasmid pMP2425 is indeed detectable with pr3 and pr8, the combination of primers for *phoE* and *caa*, whereas pMR05 shows no reaction. The controls with pr28 and pr8, both directed against *phoE*, show PCR fragments with slightly different sizes for the two plasmids, which is in agreement with the fact that pMP2425 carries a 75-bp insert which is lacking in pMR05.

To achieve the highest degree of reaction specificity of the PCR, a new primer pair was designed, allowing annealing at 50°C in an otherwise-unmodified PCR protocol. Primers pr1 and pr7, derived from pr8 and pr3, respectively, allowed reproducible detection of 5 ag of plasmid pMP2427, containing the *phoE-caa* marker gene (Fig. 8). Chromosomal DNA of six different wild-type rhizobia and of the *Pseudomonas* strains listed in Table 1 did not show the marker-specific signal in the pr1-pr7 PCR (data not shown).

DISCUSSION

Application of recombinant bacteria for agro-industrial purposes in the environment is still a matter of controversy. Since possible risks of such applications for humans and the environment are difficult to judge, monitoring of the bacteria in contained prerelease experiments, as well as in actual applications, is often required. For this purpose a wide variety of methods is used (28, 30, 37), each having its specific advantages and disadvantages. In this paper we describe the construction of a novel marker gene which can be detected by two complementary techniques, both with high degrees of sensitivity and specificity. Depending on the specific requirements of the monitoring, either MAb detection or PCR can be chosen or samples can be analyzed by both methods to virtually eliminate the chance of false-positive identification.

The marker gene is based on *E. coli phoE*, in which a 75-bp fragment of *E. coli caa* is inserted in the part of *phoE* that codes for the fourth cell surface-exposed loop of the PhoE protein (Fig. 1). This fragment codes for residues 8 to 30 of colicin A and is derived from a part of this protein which does not contain bacteriocinic activity (8). The marker thus contains only *E. coli* sequences not coding for toxins. Furthermore, since *P. putida* expresses the phosphate starvation-inducible OprP protein, introduction of *E. coli* PhoE, which has a similar function, is very unlikely to confer either a major advantage or

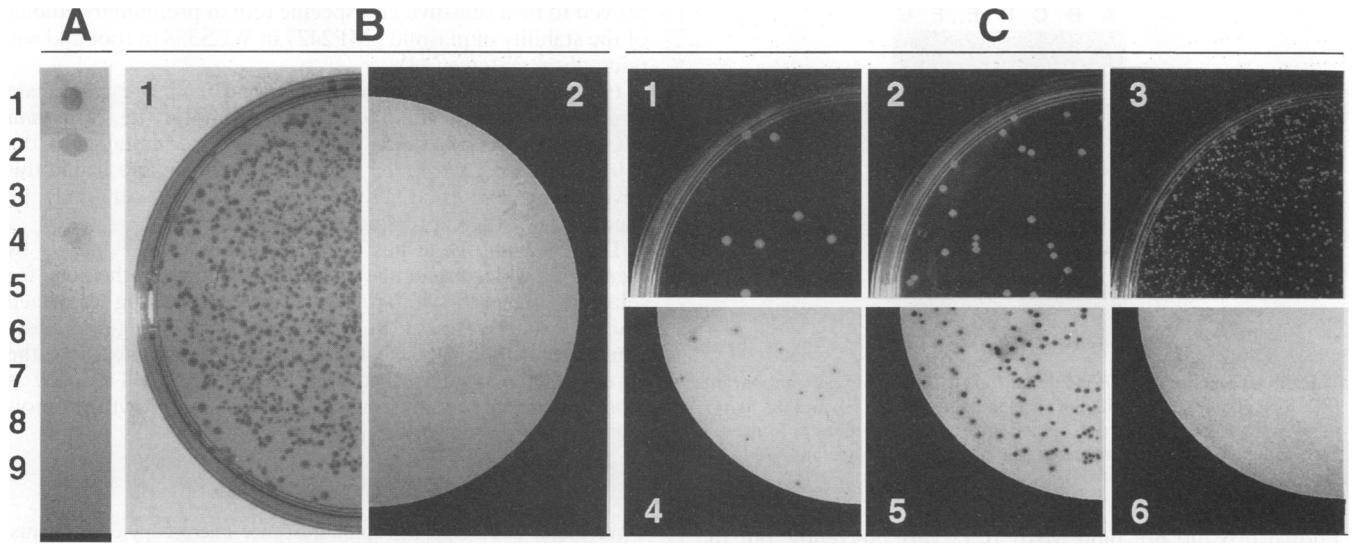

FIG. 6. Specificity of MAb 1C11. (A) Reaction of MAb 1C11 with 5-μl spots of suspensions (optical density at 620 mn of 1.0) of *Pseudomonas* strains. Blots: 1, LWP358-5c.pMP2427; 2, WCS358.pMP2427; 3, WCS358; 4, M114; 5, WCS007; 6, WCS134; 7, WCS307; 8, WCS315; 9, WCS379. (B) Microflora of 10-fold diluted extract of Flevoland potato-cropping soil (panel 1) and its reaction with MAb 1C11 (panel 2). (C) Colonies of WCS358.pMP2427 (panel 1), LWP358-5c.pMP2427 (panel 2), and WCS358 (panel 3) and their reactions with MAb 1C11 (panels 4 to 6, respectively). Photographs of bacteria before (panels C1 to C3) and after immunoblotting (panels C4 to C6) were taken from different sectors of the corresponding agar plates. In all cases, bacteria applied to nitrocellulose were washed three times for 10 min with 10 mM EDTA at 37°C prior to immunodetection.

a disadvantage to this bacterium. Therefore, the *phoE-caa* marker gene maximally meets environmental safety criteria.

The influence of amino acid insertions in the fourth cell surface-exposed loop of PhoE has been studied extensively (2–4). Insertions of up to 34 residues did not disturb correct outer membrane assembly, provided that the insert did not have an overall hydrophobic nature and that not too many positively charged residues were introduced (3). Since 23 of the 25 amino acid residues of ColA that were selected as the immunological tag are either polar or charged and since their total charge is zero, the insert meets the criteria for cell surface exposure by PhoE. Indeed, the hybrid protein is incorporated into the outer membrane of *E. coli* CE1248 cells, although the expression of the protein is reduced in comparison to that of wild-type PhoE (Fig. 2). However, in strain CE1248, expression of hybrid PhoE proteins has been previously observed to be impaired (1). In the Western blot of the membrane proteins, two bands reacting with MAb 1C11 were observed, the upper band probably being a precursor form of the mutant protein (Fig. 2B). This phenomenon has been observed with a number of other mutant PhoE proteins, carrying hydrophilic and hydrophobic inserts of various length (3). Despite the reduced expression of the hybrid protein, *E. coli* CE1248. pMP2425 cells expressing the PhoE-ColA protein could be visualized as individual cells by immunofluorescence microscopy. Since the cells were not treated with a fixative prior to immunostaining, this indicates that the ColA insert is cell surface exposed.

The PhoE protein has been used as a carrier for antigenic epitopes not only in *E. coli* but also in the closely related species *Salmonella typhimurium* (5). In this paper we show, (i) that a mutant *phoE* gene under control of its own phosphate starvation-inducible promoter is also expressed in *P. putida* under phosphate limitation, (ii) that the hybrid protein is not extracted from a membrane preparation of *P. putida* WCS358 pMP2427 by using Sarkosyl, and (iii) that it reacts with MAb 1C11 (Fig. 4). Since Sarkosyl dissolves inner membranes, the protein indeed seems to be incorporated in the outer membrane. These results extend the applicability of PhoE as a carrier for cell surface exposure of foreign epitopes to *P. putida*.

Whole cells of the wild-type *P. putida* WCS358 expressing

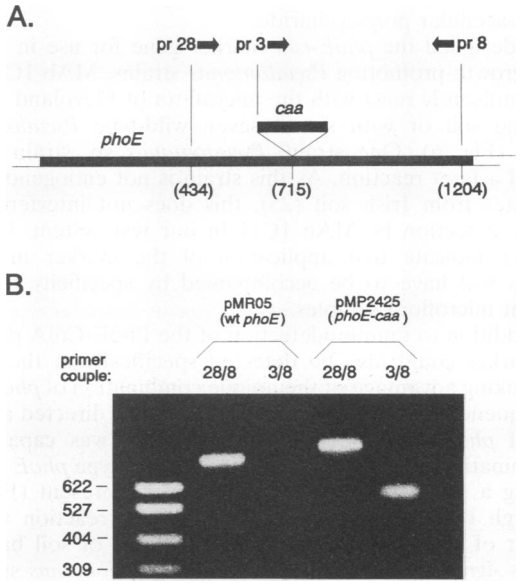

FIG. 7. Detection of *phoE-caa* marker gene by PCR. (A) Schematic representation of primers used for amplifications. (B) Agarose gel showing amplification products of various primer combinations, with pMR05 (wild-type [wt] phoE) or pMP2425 (*phoE-caa*) plasmid DNA as templates. Molecular sizes (in base pairs) are indicated on the left.

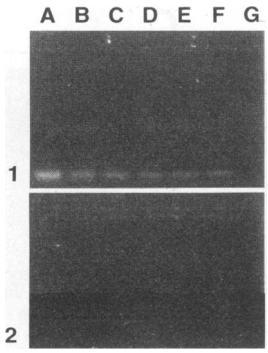

FIG. 8. Sensitivity of PCR detection of *phoE-caa*. Five nanograms (A), 5 pg (B), 50 fg (C), 500 ag (D), 50 ag (E), 5 ag (F), and 0.5 ag (G) of plasmid pMP2427 were used as templates for primers pr7 and pr1. Two independent dilution series (panels 1 and 2) were analyzed.

PhoE-ColA did not bind MAb 1C11 very efficiently, but the O-antigen mutant strains of WCS358 that expressed the hybrid protein were well stained by using MAb 1C11 in a colony blot (Fig. 5). This finding seemed to indicate that the O-antigenic side chains of the lipopolysaccharide shielded the ColA epitope on the cell surface of WCS358. The O-antigen has been described as the component responsible for shielding outer membrane proteins in *E. coli* (39, 40) as well as in *S. typhimurium* (5). Involvement of the O-antigen in the case of *P. putida* WCS358 seemed to be confirmed by the finding that washing with 10 mM EDTA prior to immunodetection rendered the cells more accessible to MAb 1C11 (Fig. 5). However, cells of WCS358 expressing PhoE-ColA that were washed with demineralized water instead of EDTA also showed some reaction with MAb 1C11. It is possible that the lipopolysaccharide is partly removed by this treatment. Alternatively, shielding may also be caused by a component(s) loosely attached to the O-antigen, which is easily washed away. A possible candidate for this component could be (small amounts of) extracellular polysaccharide.

We designed the *phoE-caa* marker gene for use in potato plant growth-promoting *Pseudomonas* strains. MAb 1C11 did not significantly react with the microflora of Flevoland potato cropping soil or with six of seven wild-type *Pseudomonas* strains (Fig. 6). One strain, *Pseudomonas* sp. strain M114 showed a faint reaction. As this strain is not endogenous but originates from Irish soil (25), this does not interfere with specific detection by MAb 1C11 in our test system. It does however indicate that application of the marker in other systems will have to be accompanied by specificity tests of relevant microflora samples.

In addition to immunodetection of the PhoE-ColA protein, the marker could also be detected specifically at the DNA level, taking advantage of the unique combination of *phoE* and *caa* sequences. A set of primers, pr3 and pr8, directed against part of *phoE* and part of the *caa* insert was capable of discriminating between the mutant and wild-type *phoE* genes, showing a signal only when the insert was present (Fig. 7). Although these primers did not show any reaction with a number of chromosomal DNA preparations of soil bacteria such as *Agrobacterium*, *Rhizobium*, and *Pseudomonas* species, we designed a set of primers, pr7 and pr1, to allow a higher annealing temperature. With these primers, 5 ag of plasmid pMP2427 could reproducibly be detected (Fig. 8), whereas chromosomal DNA of six rhizobial species and seven *Pseudomonas* species showed no specific signal. The pr1-pr7 PCR proved to be a sensitive and specific tool in preliminary studies of the stability of plasmid pMP2427 in WCS358 in root and soil microcosm systems (44).

In this study *phoE-caa* was expressed from the phosphate limitation-inducible *E. coli phoE* promoter in *P. putida* WCS358. As WCS358 experiences phosphate limitation in the rhizosphere (10), *phoE-caa* most likely will be expressed in this environment. Alternatively, constitutive promoters may be used to drive the expression of the marker gene.

The *phoE-caa* gene has been introduced into *P. putida* by means of a wide-host-range plasmid. To eliminate the need for selective pressure, we are currently constructing disarmed transposon derivatives (18) carrying the *phoE-caa* gene. These constructs will be used to stably integrate the marker into the chromosomes of selected strains. These strains will be used as model strains for monitoring the fate of plant growth-promoting pseudomonads and their DNA.

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