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Cloning and Heterologous Expression of a Gene Encoding an Alkane-Induced Extracellular Protein Involved in Alkane Assimilation from *Pseudomonas aeruginosa*

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***Pseudomonas aeruginosa* PG201 produces a 16-kDa extracellular protein in media containing *n*-hexadecane as a carbon source but not in media containing glycerol or glucose. This protein was purified, and the N-terminal amino acid sequence was determined. The amino acid composition of the protein was found to be very similar to that of the so-called protein-like activator for *n*-alkane oxidation (PA) from *P. aeruginosa* S_7B_1. This extracellular protein was previously characterized (K. Hisatsuka, T. Nakahara, Y. Minoda, and K. Yamada, Agric. Biol. Chem. 41:445–450, 1977) and found to stimulate the growth of *P. aeruginosa* on *n*-hexadecane and to possess emulsifying activity. To study the role(s) of the PA protein and to make it accessible for possible future applications, we have cloned the PA-encoding (*pra*) gene and determined its nucleotide sequence. This analysis revealed a protein-coding region of 162 amino acids, with the first 25 residues being reminiscent of those of a typical bacterial signal sequence. The *pra* gene was inactivated by insertional mutagenesis, and the resulting strain was found to lack extracellular PA protein and to be retarded in its growth in *n*-hexadecane-containing media. These results are consistent with the growth stimulatory role of the PA protein. The *pra* gene was expressed in *Escherichia coli*, and substantial amounts of the recombinant protein were found in the extracellular growth medium. The recombinant protein was purified by metal chelate affinity chromatography. The ability to produce secreted PA protein by *E. coli* provides a simple and safe means to analyze its function(s) in alkane assimilation in the future.**

A number of microorganisms satisfy their carbon and energy requirements by using compounds, such as hydrocarbons, that are poorly soluble in aqueous media. Consequently, the growth on hydrocarbons of such organisms is often associated with the production of surface-active compounds. The molecular composition, structure, and the physicochemical and biological properties of a large number of biosurfactants from various microorganisms including bacteria, yeasts, and fungi have been elucidated (for reviews, see references 11, 12, 35, 45, 46, 56). Microbial surfactants include a wide variety of chemical structures, including glycolipids, phospholipids, fatty acids, neutral lipids, lipopeptides, and lipid-containing polymers, such as lipoproteins, lipopolysaccharide-protein complexes, and polysaccharide-protein-fatty acid complexes. The most widespread microbial surfactants are glycolipids, of which the *Pseudomonas aeruginosa* rhamnolipids (11) are the best studied. Hydrophobic, protein-containing compounds from culture supernatants have been the subjects of detailed investigation concerning their possible roles as surfactants (for a recent review, see reference 7). Hisatsuka et al. (18–20) previously described the so-called protein-like activator for *n*-alkane oxidation (PA). They found that the PA together with rhamnolipids affected the stabilization of *n*-hexadecane–water emulsions. Moreover, the addition of PA to *P. aeruginosa* cell suspensions leads to an increased oxidation of the hydrocarbon substrate. The PA was purified to homogeneity by ammonium sulfate precipitation and subsequent ion-exchange chromatography and gel filtration. The purified PA was positive in protein-specific dye reactions and stimulated the oxidation of *n*-hexadecane by *P. aeruginosa* cells but not that of palmitic acid or glucose (20). Likewise, synthesis of the PA protein was observed on hydrocarbons and on cetyl alcohol but not on glucose, glycerol, or palmitic acid. PA concentrations of up to 100 mg/liter were measured after 88 h of growth in *n*-hexadecane. From the amino acid composition and gel filtration experiments, the molecular weight was estimated to be about 14,300. The PA protein was stable at low pH and high temperatures.

With a view toward making the PA protein accessible for future practical applications, we have isolated and characterized the PA-encoding (*pra*) gene and studied its expression in *Escherichia coli*.

MATERIALS AND METHODS

Strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani medium and 2× YT medium were prepared by the method of Miller (33). A nitrogen-limited minimal medium (14) containing 2% glycerol or 1% *n*-hexadecane and fortified SB medium (41) were used for monitoring PA synthesis in *P. aeruginosa* and *E. coli*, respectively. Antibiotics were applied at the following concentrations: for *E. coli*, 50 μg of kanamycin sulfate per ml, 50 μg of ampicillin per ml, 20 μg of gentamicin sulfate per ml, and 20 μg of tetracycline per ml and for *P. aeruginosa*, 100 μg of gentamicin sulfate per ml or 100 μg of tetracycline per ml.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
P. aeruginosa		
DSM2659	Referred to as PG201; prototrophic strain	13
65E12	Tn*5*-Gm^r; rhamnolipid-negative strain affected in *rhlR* gene	26, 39
UO299	Tn*5*-Gm^r; rhamnolipid-negative strain affected in *rhlA* gene	38
PG201 ('*pra*')	Strain PG201 with the *pra* gene inactivated	This study
UO299 ('*pra*')	Strain UO299 with the *pra* gene inactivated	This study
PAO1	Prototrophic strain	23
ATCC 7700	Prototrophic strain	3
PAK-N1	*rpoN* mutant of PAO1	38
P. fluorescens ATCC 15453	Prototrophic strain producing rhamnolipids	61
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	1
XL-1 Blue	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lac* F′ [*proAB lacI*^{q} *lacZ*Δ*M15* Tn*10*(Tet^r)]	2
SURE	e14-(*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)*171 sbcC recB recJ umuC*::Tn*5*(Kan^r) *supE44 lac gyrA96 relA1 thi-1 endA1* [F′ *proAB lacI*^{q} *Z*Δ*M15* Tn*10*(Tet^r)]	Stratagene Cloning Systems
Plasmids		
pBluescript SK−	*E. coli* cloning vector	53
pPA11	pBluescript SK− containing a 1.1-kb *Rsa*I fragment harboring the *pra* gene	This study
pPA6His	pPA11 with 6 His codons added to *pra* gene	This study
pSUP203	Mobilizable plasmid vector	54
pSUP203-PA	pSUP203 containing a 306-bp *pra* gene insertion	This study
pRK2013	Mobilizable helper plasmid	8
pPZ10	*lacZ*-based *Pseudomonas* promoter probe vector	52

Transformation of bacteria. Competent *E. coli* cells were prepared and transformed by the method of Chung and Miller (5) or by the Bio-Rad electroporation protocol (9). For the transformation of *P. aeruginosa*, the procedure described by Potter (40) was followed.

Isolation of the *pra* gene. For standard cloning operations, the procedures compiled by Sambrook et al. (50) were followed. To isolate the *pra* gene, total cellular DNA of *P. aeruginosa* PG201 was digested partially with *Eco*RI, and fragments in the size range between 5 and 10 kb were isolated following preparative agarose gel electrophoresis (50) and subsequently ligated to pBluescript KS− vector DNA (53), previously cut with *Eco*RI. *E. coli* DH5α cells were electroporated, and 7,000 independent colonies, 60% of which harbored recombinant plasmids, were obtained. For the isolation of the *pra* gene, a cloned 47-bp PCR fragment was labeled in a PCR mixture containing [α-^{32}P]dCTP and subsequently used as a hybridization probe in the presence of 20% formamide at 42°C. Of about 15,000 colonies screened, 13 were positive.

Disruption of the *pra* gene. To disrupt the *pra* gene, a 306-bp restriction fragment ranging from the *Nco*I site at codon −4 to the *Hin*cII site at codon +98 (see Fig. 3B) was provided with *Eco*RI linkers and subsequently cloned into pSUP203 (54) previously cut with *Eco*RI to yield pSUP203-PA. Tetracycline-resistant *P. aeruginosa* transconjugants were obtained after a triparental mating with *E. coli* DH5α/pSUP203-PA as the donor strain, *P. aeruginosa* PG201 or UO299 as the recipient, and *E. coli* HB101/pRK2013 as the mobilizing helper strain (8, 62).

Southern blot analysis. DNA from *P. aeruginosa* cells was isolated essentially as described by Koch et al. (27). DNA blotting onto GeneScreen Plus membranes (Du Pont-NEN) was carried out by the method of Southern (55) by following the protocol supplied by the manufacturer. For the preparation of radioactively labeled probes, the method of Feinberg and Vogelstein (10) was used. Hybridization was carried out in a solution containing 50% formamide, 10% dextran sulfate, and 1 M NaCl for 16 h at 42°C.

PCR. The conditions used for the synthesis of the 59-bp *pra* gene fragment included the following steps (in one cycle): 1 min at 95°C; 2 min at 48°C; and 15 s at 72°C. After 30 cycles, an incubation step at 72°C for 2 min was carried out. PG201 DNA (100 ng) and 10 pmol of the oligonucleotides, were used in a 100-μl reaction volume together with 1.5 U of *Taq* polymerase (Boehringer Mannheim) and the buffer provided by the supplier. Deoxynucleotides at 200 μM each were used. The PCR product of interest was cloned by the procedure described by Jung et al. (24). For labeling experiments, the concentration of the unlabeled dCTP was 0.8 μM, and 2 μl of [α-^{32}P]dCTP (3,000 Ci/mmol; 10 mCi/ml) was added to the reaction mixture. For PCR amplification of the *pra* promoter fragment, 10 μg of each primer, 10 ng of pPA11 plasmid DNA, 10 μl of a 2 mM deoxynucleoside triphosphate solution, 10 μl of 10× *Taq* polymerase buffer (100 mM Tris-HCl [pH 9.0], 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100, 0.1% gelatin; ANAWA, Wangen, Switzerland), and 2.5 U of *Taq* polymerase (ANAWA) were combined, and the volume was adjusted to 100 μl. One cycle consisted of 1.5 min of denaturation at 94°C, annealing for 2 min at 50°C, and polymerization for 2 min at 72°C. This cycle was repeated 30 times. After a 10-min incubation at 72°C, 10 μg of carrier glycogen (Boehringer Mannheim) was added and the reaction product was extracted with 100 μl of a 1:1 mixture of phenol and chloroform. The DNA was precipitated with ethanol and subsequently digested with *Sal*I and *Pst*I and fractionated on a low-melting-temperature agarose gel and subsequently ligated to pPZ10 vector DNA previously cut with *Sal*I and *Pst*I primer 1 (5′ AGCGGA TAACAATTTCACACAGGA) and primer 2 (5′ GGCGCGC CTGCAGGATTTCATTGTTTTGTA) were used.

DNA sequencing. The sequencing reactions with Sequenase

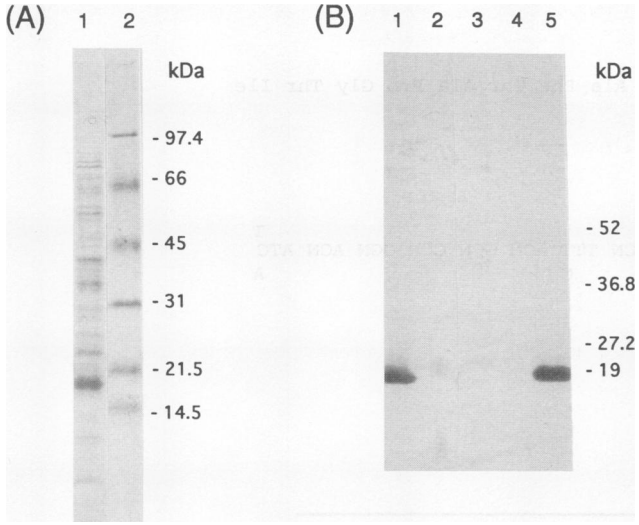


FIG. 1. Extracellular proteins of *n*-hexadecane-grown *P. aeruginosa* wild-type and mutant cells. Concentrated cell supernatants were applied to a SDS–Tricine–10% polyacrylamide gel. (A) Coomassie brilliant blue-stained gel. Lane 1, concentrated cell-free supernatant of a *n*-hexadecane-grown PG201 culture; lane 2, molecular mass markers. (B) Western blot. Lanes: 1, concentrated cell-free supernatant of a *n*-hexadecane-grown culture of the PG201 wild-type strain; 2, concentrated cell-free supernatant of a glycerol-grown culture of the PG201 wild-type strain; 3, concentrated cell-free supernatant of a *n*-hexadecane-grown culture of the PG201 strain carrying the truncated and inactivated '*pra*' gene; 4, concentrated cell-free supernatant of a *n*-hexadecane-grown culture of the non-rhamnolipid-producing UO299 mutant strain carrying '*pra*'; 5, concentrated cell-free supernatant of a *n*-hexadecane-grown culture of the non-rhamnolipid-producing UO299 mutant strain.

TABLE 2. Amino acid composition of PA proteins

Amino acid	No. of amino acids in PA protein		
	P. aeruginosa PG201	*P. aeruginosa* S_7B_1[a]	Inferred from gene sequence[b]
Ala	12	12	12
Arg	0	0	0
Asx (Asp and/or Asn)	14	14	14
Cys	+[c]	4	4
Glx (Glu and/or Gln)	10	2	2
Gly	11	11	11
His	0	0	0
Ile	6	6	6
Leu	9	9	9
Lys	7	7	2.0
Met	+	1	1
Phe	4	4	4
Pro	9	10	10
Ser	19	32	23
Thr	16	19	18
Trp	ND[d]	2	2
Tyr	1	1	1
Val	13	13	13

[a] Adapted from reference 19.
[b] Adapted from DNA sequence shown in Fig. 3B.
[c] +, detected.
[d] ND, not determined.

(version 2.0; United States Biochemicals) were performed by the method of Tabor and Richardson (57), using [^{35}S]dATP following the instructions provided by the supplier, and analyzed on 6% polyacrylamide sequencing gels.

Preparation of extracellular proteins from *E. coli* and *P. aeruginosa*. *E. coli* SURE cells harboring pPA6His were grown in fortified SB medium, and extracellular proteins were precipitated overnight at 4°C with ammonium sulfate (55 g/100 ml of supernatant). After centrifugation, the pellet was dissolved in deionized water (5 ml/100 ml of the original culture). One liter of *P. aeruginosa* cells was grown in a *n*-hexadecane-containing medium for 120 h. After centrifugation, ammonium sulfate was added to the supernatant by stirring to a final concentration of 4 M. The solution was left at 4°C overnight and then centrifuged (6,000 × *g*) at 4°C for 1 h. The pellet was dissolved in 5 ml of water and dialyzed against deionized water for 24 h with five changes of 5 liters each.

Purification of recombinant PA from *E. coli* by metal chelate affinity chromatography. A Ni^{2+}-nitrilotriacetic acid (NTA)-agarose column (Quiagen; 2.5 ml) was set up and equilibrated in 15 ml of BC buffer (20 mM Tris-HCl [pH 7.9], 20% glycerol, 0.2 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride) containing 100 mM KCl and 40 mM imidazole (BC 100/40) (59). Five milliliters of the redissolved ammonium sulfate precipitate was diluted with 7.5 ml of BC 100/40 buffer and applied to the column. The column was washed with 12.5 ml of BC 100/40, and then PA was eluted with 7.5 ml of BC containing 1 M KCl and 100 mM imidazole (BC 1000/100) and 7 ml of BC containing 1 M KCl and 2 M imidazole (BC 1000/2000). The BC 1000/100 pool was desalted on a PD10 column (Pharmacia). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the eluate was concentrated by using StrataClean resin (Stratagene Cloning Systems) (36).

SDS-PAGE of proteins. SDS-PAGE was performed in slab gels, using the discontinuous buffer system of Laemmli (30) with a acrylamide gradient of 10 to 30% in the separating gel. Alternatively, the gel system of Schägger and von Jagow (51) was used. Cell-free culture supernatants were concentrated by using StrataClean resin.

Protein sequence analysis. For preparative SDS-PAGE, 2 ml of a dialyzed protein solution was mixed with an equal volume of 2× SDS sample buffer (30) and warmed at 60°C for 12 min. Five hundred sixty micrograms of protein was loaded per gel. The proteins were separated at 250 V for 4 h at room temperature. Proteins to be eluted from the gel were stained for only 5 to 10 min and destained in 30% methanol–10% acetic acid for 15 min at room temperature. The proteins were electroeluted as previously described (32), and the precipitate which was obtained after electroelution was dissolved in 500 μl of 100% formic acid and then diluted with an equal volume of water. A 600-μl aliquot was desalted on a Biogel P2 column. One part of the sample was hydrolyzed with boiling hydrochloric acid for 48 h and then applied to an amino acid analyzer. The other part of the desalted sample was used for the amino acid sequence analysis in a model 470 A gas phase sequencer (Applied Biosystems, Foster City, Calif.).

Preparation of antibodies. One hundred micrograms of gel-purified protein in 400 μl of 0.1% SDS were mixed with 400 μl of complete Freund's adjuvant and injected subcutaneously into New Zealand White rabbits. Booster injections were given by following the schedule described by Renart et al. (48).

Western blot (immunoblot) analysis of proteins. Proteins were separated by SDS-PAGE (51) and either stained with Coomassie blue or transferred electrophoretically onto a nitrocellulose filter (Schleicher and Schuell) by using 15 mM sodium phosphate buffer, pH 6.5, as described by Reiser and

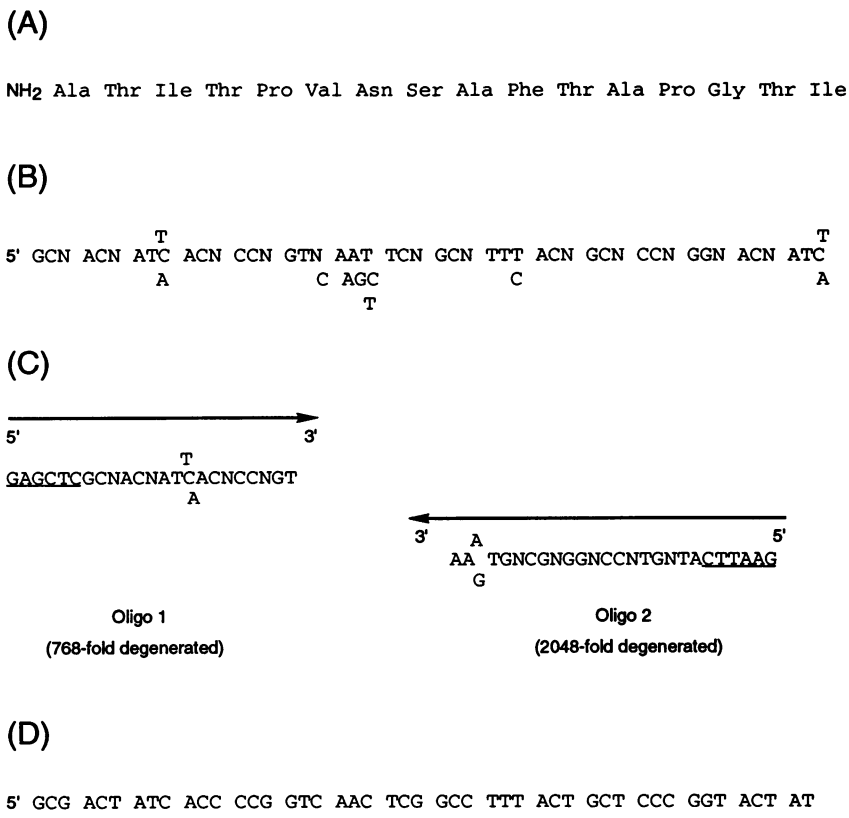


FIG. 2. Preparation of a PA-specific gene probe using the PCR. (A) N-terminal amino acid sequence of the PA protein; (B) derived nucleotide sequences; (C) PCR primers used. N stands for any of the four nucleotides. The two underlined sequences constitute *Sac*I and *Eco*RI sites, which have been added to facilitate cloning of the PCR product. Oligo, oligonucleotide. (D) nucleotide sequence of the cloned PCR product. Note that the linker sequences are not shown.

Stark (47). After the transfer, the filters were blocked in Tris-buffered saline (25 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 5% Bacto skim milk (Difco) for 1 h at room temperature. The primary rabbit anti-PA antibody was diluted 1:1,000 into Tris-buffered saline containing 5% Bacto skim milk and 0.05% Tween 20. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) diluted 1:1,000 in the above buffer was used as the secondary antibody. In situ peroxidase activity was detected by using the conditions described by Hawkes et al. (17).

Determination of β-galactosidase activity. β-Galactosidase activity was measured by following the standard *o*-nitrophenyl-β-D-galactopyranoside protocol (50).

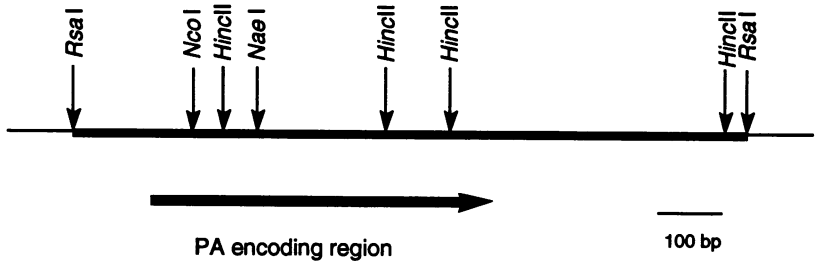
Nucleotide sequence accession number. The nucleotide sequence reported for the *pra* gene in this study has been entered in the GenBank data library and assigned accession no. L08966.

RESULTS

Isolation and analysis of a *n*-hexadecane-induced extracellular protein from *P. aeruginosa* PG201. The *P. aeruginosa* PG201 and PAO1 strains produced a number of ammonium sulfate-precipitable extracellular proteins when cultivated in a medium containing *n*-hexadecane as a carbon source. One of the proteins with a molecular mass of roughly 16 kDa represented up to 50% of the extracellular protein fraction of the *P. aeruginosa* PG201 strain, as judged from SDS-PAGE analysis (Fig. 1A, lane 1) and subsequent densitometric scanning. This protein was purified by preparative SDS-PAGE and injected into rabbits to generate specific antibody. The Western blot analysis presented in Fig. 1B shows that the generated antiserum detected a protein of about 16 kDa in the culture supernatants of the *P. aeruginosa* PG201 and UO299 strains, previously grown in a medium containing *n*-hexadecane as the carbon source (Fig. 1B, lanes 1 and 5). In cultures containing glycerol or glucose as the carbon source, this protein was absent (lane 2). To investigate the gel-purified 16-kDa protein in more detail, an analysis of the N-terminal amino acids was carried out and the amino acid composition was determined. The sequence of the first 27 amino acids was as follows: ATITPVNSAFTAPGTISVsxPAxLtLP, with x representing an unknown amino acid and lowercase letters indicating ambiguities in the analysis. The amino acid composition of the protein was found to be very similar to that of the so-called PA (19). The amino acid composition of the PA is striking, in that 30% of the residues are Ser or Thr and Arg and His are absent (Table 2).

Isolation of the PA-encoding gene. On the basis of the first 16 amino acids of the PA protein (Fig. 2A), two mixtures of highly degenerated oligonucleotides were synthesized (Fig. 2C) and subsequently used in a PCR to generate a specific probe for the *pra* gene. After 30 cycles of amplification using *P. aeruginosa* PG201 DNA as a template, the resulting 59-bp PCR product was treated with *Sac*I and *Eco*RI and subsequently ligated to pBluescript SK− vector DNA previously treated with *Sac*I and *Eco*RI. One of the clones contained a 47-bp insert of *P. aeruginosa* DNA corresponding to the N-terminal

(A)



(B)

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ATCGGACGGGAAGGCACAAGGTGGTCGTGGGGGCGGGTCGGCGACGATCCTGCCGC
ATGATCAAGGCTCGGCAACCGCGAGCGTTGCCAACTCACTGGAAGGATACAAAACA
-25
ATG AAA TCC ATC AAG TCC CTG CCG TCC TTT GCT GCT CTT GCT
Met Lys Ser Ile Lys Ser Leu Pro Ser Phe Ala Ala Leu Ala
                  NcoI                  -1  +1
CTC TGC CTG AGC GTT TCG TC↓C ATG GCC AGC GCG GCC ACC ATC
Leu Cys Leu Ser Val Ser Ser  Met Ala Ser Ala Ala Thr Ile
       HincII          +10
ACC CCG GTC↓AAC TCG GCC TTC ACC GCA CCT GGC ACC ATC AGC
Thr Pro Val Asn Ser Ala Phe Thr Ala Pro Gly Thr Ile Ser
      +20                                          +30
GTG AGC TCG CCG GCG TCG CTC AAT CTG CCA GTG ACC TGC AAC
Val Ser Ser Pro Ala Ser Leu Asn Leu Pro Val Thr Cys Asn
                           +40
ATC ACC TTC AAG GGC AAG ACC GCC GCC GAT GGT TCC TAT GCC
Ile Thr Phe Lys Gly Lys Thr Ala Ala Asp Gly Ser Tyr Ala
            +50
TCG ATC GAC AGC GTC ACC GTC AGC GGC AGC AAC ACC CTG TGC
Ser Ile Asp Ser Val Thr Val Ser Gly Ser Asn Thr Leu Cys
+60                                          +70
AGC GTG CCG CAG ATG ACC GGC CTG CCG TGG AAG CTG ACC GTT
Ser Val Pro Gln Met Thr Gly Leu Pro Trp Lys Leu Thr Val
                     +80
TCC AGC ACC ACT GCC GGC AAG GTC GAT GGC GTC GGC TTC AAG
Ser Ser Thr Thr Ala Gly Lys Val Asp Gly Val Gly Phe Lys
                                    HincII
      +90                                    +100
ATT CTC TCC TCT ACC TGT GGC CCG AGC ACC GTC↓AAC GGT TCC
Ile Leu Ser Ser Thr Cys Gly Pro Ser Thr Val Asn Gly Ser
                        +110
TGG AGC AAC GCC ACC AAT ACC CTC AGC GCC AGC AAT CAG TCG
Trp Ser Asn Ala Thr Asn Thr Leu Ser Ala Ser Asn Gln Ser
          +120
CTG GCA GGC AAC TGC AAG ATC AAC AGC CTG AGC GTG AAG CCG
Leu Ala Gly Asn Cys Lys Ile Asn Ser Leu Ser Val Lys Pro
+130          HincII
ACC CCG GCC TTC GTA GTC↓AAC CCG TAA TCCGGGCTGCCGCT
Thr Pro Ala Phe Val Val Asn Pro *
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FIG. 3. Molecular analysis of the *pra* gene. (A) Restriction map. (B) Nucleotide sequence and the inferred amino acid sequence. The signal sequence is shown in bold letters.

amino acid sequence (Fig. 2D). The labeled insert fragment was then used as a probe to screen a *P. aeruginosa* PG201 gene library. Several of the hybridization-positive clones harbored recombinant plasmids with a 6.0-kb *Eco*RI insert of *P. aeruginosa* DNA (15).

Analysis of the *pra* gene. The 6.0-kb *Eco*RI insert fragment was analyzed further by Southern blot analysis, and a 1.1-kb *Rsa*I subfragment was cloned into the *Eco*RV site of the pBluescript SK− vector to yield plasmid pPA11. The DNA sequence analysis of the pPA11 insert fragment revealed an open reading frame of 486 bp corresponding to 162 amino acids (Fig. 3A). The DNA sequence of the *pra* gene and the deduced amino acid sequence are shown in Fig. 3B. The first 25 residues show the features of a typical signal sequence (22). The mature PA protein is composed of 137 amino acids, and its size, calculated from the sum of the molecular masses of the amino acids, is 16.4 kDa. A typical Shine-Dalgarno sequence (GGAAGG) is located 11 to 16 nucleotides upstream of the translation start codon. The G+C content of the *pra* gene is 63.2% and agrees well with that of other *P. aeruginosa* chromosomal genes (63). At the third position of the codon, G or C are found in 85% of the cases. The amino acid composition inferred from the nucleotide sequence corresponds well with the values obtained from the protein analysis (Table 2). The *pra* sequence did not match any of the known sequences present in the GenBank and EMBL data libraries (15).

The labeled *pra* gene fragment hybridized to a 6.0-kb *Eco*RI fragment present in the DNA of the *P. aeruginosa* UO299 strain (Fig. 4A, lane 1). Hybridization to a 6.0-kb *Eco*RI fragment in the DNAs of the *P. aeruginosa* PG201, PAO1, and ATCC 7700 strains was also observed, while no hybridization

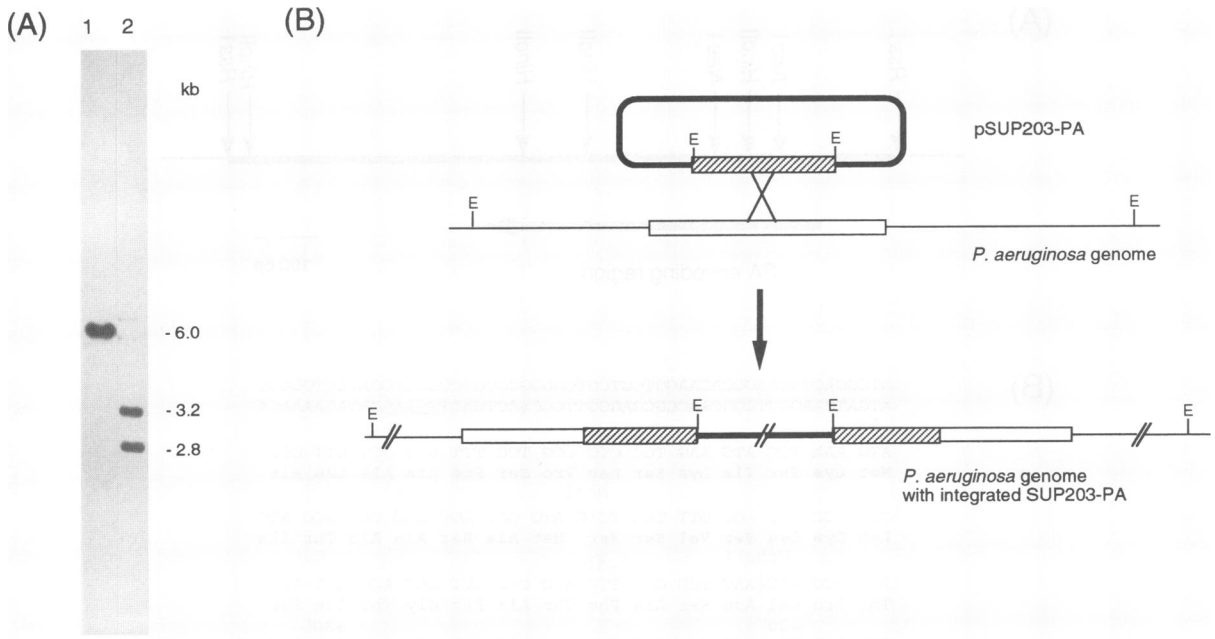

FIG. 4. Disruption of the *pra* gene. (A) Southern blot analysis of *pra* gene sequences. Lanes: 1, DNA of the *P. aeruginosa* UO299 mutant strain; 2, DNA of the UO299 mutant strain carrying the truncated and inactivated '*pra*' gene. The DNAs were cleaved with *Eco*RI and analyzed in a 0.8% agarose gel. A ^{32}P-labeled, 306-bp restriction fragment extending from the *Nco*I site at codon −4 to the *Hin*cII site at codon +98 was used as a hybridization probe. (B) Schematic diagram of the events leading to the hybridization pattern observed in the Southern blot. The hatched areas represent plasmid-derived *pra* gene sequences. Integration of pSUP203-PA at the homologous site in the *P. aeruginosa* genome leads to the disappearance of the 6-kb *Eco*RI fragment present in UO299 DNA (lane 1 of panel A) and to the appearance of two new *Eco*RI fragments of 3.2 and 2.8 kb, respectively (lane 2 of panel A), the relative location of which has not been determined. Identical hybridization patterns were observed with the DNAs of four independent transformants (44).

was seen with DNA from the *Pseudomonas fluorescens* ATCC 15453 strain (44).

To unequivocally prove that the 16-kDa protein is encoded by the *pra* coding region, the *pra* gene was inactivated by a plasmid insertion strategy (39), as depicted in Fig. 4B. As predicted, the protein was absent in two *P. aeruginosa* mutant strains in which the *pra* gene had been inactivated (Fig. 1B, lanes 3 and 4).

Expression of the *pra* gene in *E. coli*. With a view toward examining the production of PA in heterologous hosts, the expression of the cloned *pra* gene in *E. coli* was investigated. The results obtained with the *pra* promoter fragment fused to a *lacZ* reporter gene present on plasmid pPZ10 (52) are consistent with the view that the *pra* gene promoter is read efficiently in *E. coli* (44). To test PA production in heterologous hosts, plasmid pPA11 was introduced into the *E. coli* XL1-Blue and SURE strains and cell fractionation studies were carried out. Substantial amounts of a roughly 16-kDa protein which cross-reacted with an anti-PA antibody in Western blots were found in the concentrated culture supernatants (15). To demonstrate more directly the correlation between the *E. coli*-produced 16-kDa protein and the *pra* gene, the recombinant plasmid was engineered to include six histidine residues at the carboxy terminus of the PA protein to yield plasmid pPA6His (Fig. 5A). Supernatants of *E. coli* SURE cultures harboring the engineered plasmid were concentrated by ammonium sulfate precipitation, and the proteins were applied to a Ni^{2+}-NTA-agarose column (21). In the presence of 40 mM imidazole, most of the soluble bacterial proteins did not bind to the Ni^{2+}-NTA column, whereas a significant portion of the PA6His fusion protein was retained, as judged from the SDS gel analysis shown in Fig. 5B, lanes 2 and 3. The BC 1000/100 step released substantial amounts of the fusion protein(s) (Fig. 5B, lane 4), and a final wash of BC 1000/2000 eluted the remaining 10% of the fusion protein. This analysis shows that the protein observed in the culture supernatants is encoded by the *pra* gene. Compared with the PA protein from *P. aeruginosa*, the *E. coli*-derived protein had slightly less mobility. This difference is most likely due to the 10 additional amino acids at the carboxy terminus. Also, two bands differing in their electrophoretic mobilities and intensities were visible on the gel. The lower band may have arisen from the upper one, because of proteolytic breakdown. Up to 250 μg of highly purified PA fusion protein was obtained per 100 ml of culture.

Functions of the PA protein. To investigate the role(s) of the PA-encoded protein in vivo, the growth in a *n*-hexadecane-containing medium of the *P. aeruginosa* PG201 wild-type strain, the rhamnolipid-deficient *P. aeruginosa* UO299 mutant strain, and the PG201 and UO299 strains carrying the truncated and inactivated '*pra*' gene was investigated. Since the UO299 strain lacks extracellular rhamnolipids (38), its growth in *n*-hexadecane-containing media is impaired relative to that of the PG201 wild-type strain (Fig. 6) and the growth of the PG201 and UO299 strains carrying the truncated '*pra*' gene lacking extracellular PA was slowed down further relative to their counterparts harboring an intact *pra* gene (Fig. 6). These results underline the importance of rhamnolipids and PA in *n*-hexadecane assimilation.

DISCUSSION

The results presented in this work show that the *P. aeruginosa* PG201 strain produces a PA protein which is structurally

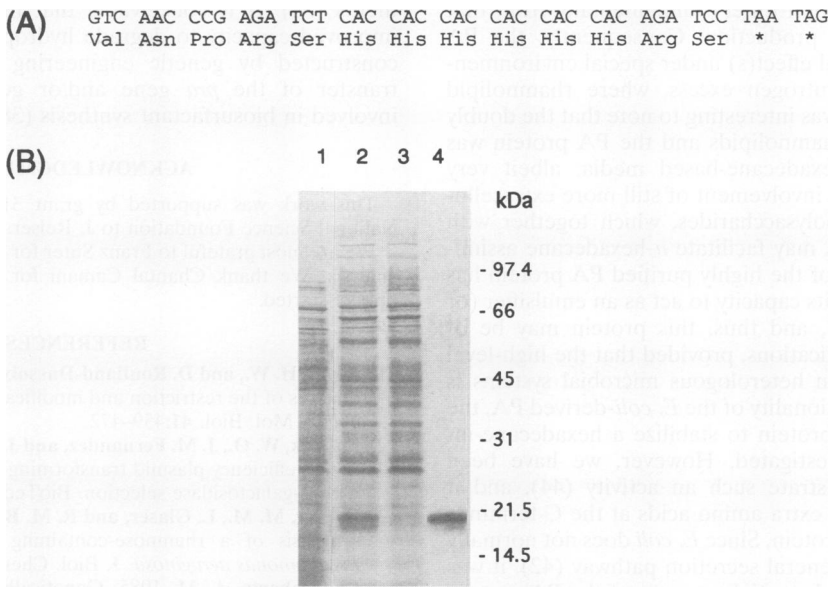

FIG. 5. Expression of the *pra* gene in *E. coli* SURE cells and purification of the recombinant protein by Ni^{2+}-NTA-agarose chromatography. (A) 3′-end of the protein-coding region of the modified *pra* gene and the corresponding amino acid sequence. The 3′ end of the gene was modified with synthetic oligonucleotides. A description of the cloning steps followed will be provided upon request. (B) Purification of the PA6His fusion protein by Ni^{2+}-NTA-agarose chromatography. The cells had been grown for 24 h in fortified SB medium at 37°C. Lanes: 1, concentrated supernatant of a control strain lacking pPA6His; 2, column input, *E. coli* SURE/pPA6His supernatant; 3, column flowthrough; 4, concentrated BC 1000/100 eluate. The samples were analyzed on a SDS–Tricine–10% polyacrylamide gel.

very similar to that of the *P. aeruginosa* S_7B_1 strain which has previously been characterized in detail by Hisatsuka et al. (18–20). The inactivation of the *pra* gene through homologous recombination has resulted in strains which are impaired in their growth in media containing *n*-hexadecane as a carbon source. These results are consistent with the in vitro findings of Hisatsuka et al. (18–20) and show that rhamnolipids and the extracellular PA protein participate in *n*-hexadecane assimilation (26, 38, 39). However, compared with the effect(s) exerted by the rhamnolipids, the PA protein had a less dominating role in hexadecane assimilation as far as the above experimental conditions are concerned. The finding that PA production was unaltered in the 65E12 mutant strain (44), which lacks a pleiotropic transcriptional activator involved in the control of rhamnolipid biosynthesis (39), and in a *rpoN* mutant strain lacking a σ^{54}-type RNA polymerase (38) indicates that the

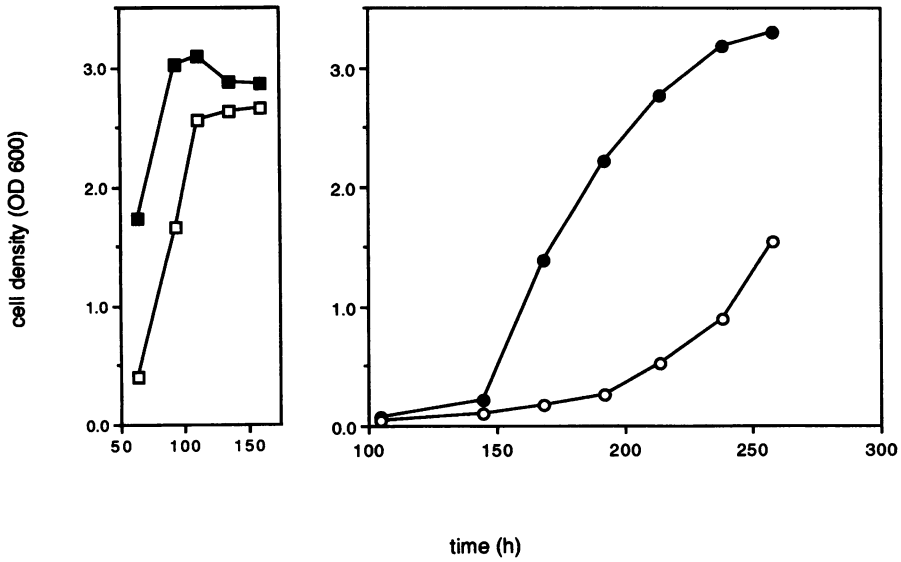

FIG. 6. Growth of the *P. aeruginosa* PG201 and UO299 strains carrying intact and truncated (inactivated) *pra* genes in a *n*-hexadecane-containing minimal medium. The cultures were incubated at 30°C. Symbols: ■, PG201 (*pra*); □, PG201 (*'pra'*); ●, UO299 (*pra*); ○, UO299 (*'pra'*). (*pra* refers to intact *pra* genes, and *'pra'* refers to inactivated [truncated] ones.) OD 600, optical density at 600 nm.

regulation of PA production differs fundamentally from that involved in rhamnolipid production. Consequently, the PA may exert its physiological effect(s) under special environmental conditions such as nitrogen excess, where rhamnolipid production is shut off. It was interesting to note that the doubly mutated strain lacking rhamnolipids and the PA protein was still able to grow in hexadecane-based media, albeit very slowly. This points to the involvement of still more extracellular factors such as lipopolysaccharides, which together with rhamnolipids and the PA may facilitate *n*-hexadecane assimilation (37, 39). The role of the highly purified PA protein has been shown to be due to its capacity to act as an emulsifier (or emulsion stabilizer) (19), and thus, this protein may be of interest for practical applications, provided that the high-level expression of the gene in heterologous microbial systems is possible. To test the functionality of the *E. coli*-derived PA, the capacity of the purified protein to stabilize a hexadecane-in-water emulsion was investigated. However, we have been unable so far to demonstrate such an activity (44), and it remains to be seen if the extra amino acids at the C terminus affect the activity of the protein. Since *E. coli* does not normally secrete proteins by the general secretion pathway (42), it was interesting to note that substantial amounts of the PA protein were present in the growth medium. At this point however, we cannot rule out the possibility that this is due to nonspecific leakage of the PA protein from the periplasmic space, although the plasmid-encoded β-lactamase appears to have been retained in the periplasm.

A number of proteins which are amphiphilic and which partition preferentially at interfaces have been analyzed (for a recent review, see reference 46). Perhaps the most common amphiphilic structure is the peptide α-helix in which hydrophobic and hydrophilic amino acids are arranged at opposite faces. The identification of α-helical structures and/or amphiphilic β-strand-forming sequences within the PA protein through structural and functional studies will depend on the availability of large amounts of highly purified protein, and our simplified purification procedure may facilitate such studies in the future.

The initial focus and most of the industrial interest in biosurfactants so far has been toward applications for microbe-enhanced oil recovery and various other uses in the oil service industry (4). However, applications in cosmetics, food, and beverages and for depollution and in environmental control may follow (for recent reviews, see references 25, 34, 49, and 60), provided that the physiology, genetics, and biochemistry of the biosurfactant-producing organisms are better understood (11). A number of polymeric biosurfactants from microbial cells consisting of protein and carbohydrate and/or lipid have been described (7). Among these, the PA protein (18–20) is by far the best characterized in terms of structure and function. Koronelli et al. (28, 29) have described a peptidoglycolipid from *P. aeruginosa* P-20 which is capable of emulsifying hydrocarbons. This bioemulsifier bears 52 amino acids (Ser being the predominant one), fatty acids, and rhamnose. The relationship between the PA protein and this bioemulsifier remains to be seen. Protein-containing bioemulsifiers from other *Pseudomonas* strains including the *P. aeruginosa* UG1 strain (31), a *P. fluorescens* isolate (6), and from a *Pseudomonas* species (43) have also been described. The exact chemical structures of these emulsifiers still need to be elucidated.

The microbial decontamination of soil is an upcoming potential application for biosurfactants (16, 34). The *P. aeruginosa* biosurfactants have recently been shown to be very efficient in the remediation of hydrophobic pollutants such as hydrocarbons and polychlorinated biphenyls in soil environments (58). It is conceivable that microbial strains with an improved capacity to degrade hydrophobic pollutants can be constructed by genetic engineering in the future through transfer of the *pra* gene and/or genes encoding enzymes involved in biosurfactant synthesis (38, 39).

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REFERENCES

1. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
2. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with β-galactosidase selection. BioTechniques **5**:376–379.
3. **Burger, M. M., L. Glaser, and R. M. Burton.** 1963. The enzymatic synthesis of a rhamnose-containing glycolipid by extracts of *Pseudomonas aeruginosa*. J. Biol. Chem. **238**:2595–2602.
4. **Chakrabarty, A. M.** 1985. Genetically-manipulated microorganisms and their products in the oil service industries. Trends Biotechnol. **3**:32–38.
5. **Chung, C. T., and R. H. Miller.** 1988. A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res. **16**:3580.
6. **Desai, A. J., K. M. Patel, and J. D. Desai.** 1988. Emulsifier production by *Pseudomonas fluorescens* during the growth on hydrocarbons. Curr. Sci. **57**:500–501.
7. **Desai, J. D., and A. J. Desai.** 1993. Production of biosurfactants, p. 65–97. *In* N. Kosaric (ed.), Biosurfactants: production, properties, applications. Marcel Dekker Inc., New York.
8. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
9. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16**:6127.
10. **Feinberg, P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266–267.
11. **Fiechter, A.** 1992. Biosurfactants: moving towards industrial application. Trends Biotechnol. **10**:208–217.
12. **Georgiou, G., S. C. Lin, and M. M. Sharma.** 1992. Surface-active compounds from microorganisms. Bio/Technology **10**:60–65.
13. **Guerra-Santos, L. H., O. Käppeli, and A. Fiechter.** 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl. Environ. Microbiol. **48**:301–305.
14. **Guerra-Santos, L. H., O. Käppeli, and A. Fiechter.** 1986. Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Appl. Microbiol. Biotechnol. **24**:443–448.
15. **Hardegger, M.** Unpublished data.
16. **Harvey, S., I. Elashvili, J. J. Valdes, D. Kamely, and A. M. Chakrabarty.** 1990. Enhanced removal of Exxon Valdez spilled oil from Alaskan gravel by a microbial surfactant. Bio/Technology **8**:228–230.
17. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**: 142–147.
18. **Hisatsuka, K., T. Nakahara, Y. Minoda, and K. Yamada.** 1975. Capacity to oxidize n-alkane in EDTA-treated cells of *Pseudomonas aeruginosa* S_7B_1. Agric. Biol. Chem. **39**:999–1005.
19. **Hisatsuka, K., T. Nakahara, Y. Minoda, and K. Yamada.** 1977. Formation of protein-like activator for n-alkane oxidation and its properties. Agric. Biol. Chem. **41**:445–450.
20. **Hisatsuka, K., T. Nakahara, and K. Yamada.** 1972. Protein-like

activator for n-alkane oxidation by *Pseudomonas aeruginosa* S_7B_1. Agric. Biol. Chem. **36**:1361–1369.
21. **Hochuli, E., W. Bannwarth, H. Döbeli, R. Gentz, and D. Stüber.** 1988. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. Bio/Technology **6**:1321–1326.
22. **Holland, B. I., N. Mackman, and J. Nicaud.** 1986. Secretion of proteins from bacteria. Bio/Technology **4**:427–431.
23. **Holloway, B. W.** 1969. Genetics of *Pseudomonas*. Bacteriol. Rev. **33**:419–443.
24. **Jung, V., S. B. Pestka, and S. Pestka.** 1990. Efficient cloning of PCR generated DNA containing terminal restriction endonuclease recognition sites. Nucleic Acids Res. **18**:6156.
25. **Klekner, V., and N. Kosaric.** 1993. Biosurfactants for cosmetics, p. 373–389. *In* N. Kosaric (ed.), Biosurfactants: production, properties, applications. Marcel Dekker Inc., New York.
26. **Koch, A. K., O. Käppeli, A. Fiechter, and J. Reiser.** 1991. Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. J. Bacteriol. **173**:4212–4219.
27. **Koch, A. K., J. Reiser, O. Käppeli, and A. Fiechter.** 1988. Genetic construction of lactose-utilizing strains of *P. aeruginosa* and their application in biosurfactant production. Bio/Technology **6**:1335–1339.
28. **Koronelli, T. V., T. I. Komarova, and Y. V. Denisov.** 1983. Chemical composition and role of peptidoglycolipid of *Pseudomonas aeruginosa* in the assimilation of hydrocarbons. Mikrobiologiya **52**:767–770.
29. **Koronelli, T. V., T. I. Komarova, and A. V. Iznatchenko.** 1983. The role of emulsification in the process of hydrocarbon absorption by cells of *Pseudomonas aeruginosa*. Mikrobiologiya **52**:94–97.
30. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**: 680–685.
31. **MacElwee, C. G., H. Lee, and J. T. Trevors.** 1990. Production of extracellular emulsifying agent by *Pseudomonas aeruginosa* UG1. J. Ind. Microbiol. **5**:25–32.
32. **McDonald, C., S. Fawell, D. Pappin, and S. Higgins.** 1986. Electroelution of proteins from SDS gels. Trends Genet. **2**:35.
33. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Müller-Hurtig, R., F. Wagner, R. Blaszczyk, and N. Kosaric.** 1993. Biosurfactants for environmental control, p. 447–469. *In* N. Kosaric (ed.), Biosurfactants: production, properties, applications. Marcel Dekker Inc., New York.
35. **Mulligan, C. N., and B. F. Gibbs.** 1993. Factors influencing the economics of biosurfactants, p. 329–371. *In* N. Kosaric (ed.), Biosurfactants: production, properties, applications. Marcel Dekker Inc., New York.
36. **Nielson, K., M. Ehret, and E. Mathur.** 1993. Concentration of dilute protein solutions with StrataClean™ resin. Strategies Mol. Biol. **6**:29.
37. **Ochsner, U. A.** 1993. Ph.D. thesis, Swiss Federal Institute of Technology, Zürich.
38. **Ochsner, U. A., A. Fiechter, and J. Reiser.** 1994. Isolation, characterization and expression in *Escherichia coli* of the *Pseudomonas aeruginosa rhlAB* genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. J. Biol. Chem. **269**:19787–19795.
39. **Ochsner, U. A., A. K. Koch, A. Fiechter, and J. Reiser.** 1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **176**:2044–2054.
40. **Potter, A. A.** 1985. Gene cloning in *Pseudomonas aeruginosa*, p. 147–156. *In* J. A. Dillon, A. Nasim, and E. R. Nestman (ed.), Recombinant DNA methodology. John Wiley and Sons, New York.
41. **Power, B. E., N. Ivanic, V. R. Harley, R. G. Webster, A. Kortt, R. A. Irving, and P. J. Hudson.** 1992. High-level temperature-induced synthesis of an antibody VH-domain in *Escherichia coli* using the PelB secretion signal. Gene **113**:95–99.
42. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57**:50–108.
43. **Reddy, P. G., H. D. Singh, M. G. Pathak, S. D. Bhagat, and J. N. Baruah.** 1983. Isolation and functional characterization of hydrocarbon emulsifying and solubilizing factors produced by a *Pseudomonas* species. Biotechnol. Bioeng. **25**:387–401.
44. **Reiser, J.** Unpublished data.
45. **Reiser, J., A. K. Koch, K. Jenny, and O. Käppeli.** 1989. Structure, properties, and production of biosurfactants, p. 85–97. *In* J. W. Obringer and H. S. Tillinghast (ed.), Advances in biotechnology for aerospace applications. The Portfolio Publishing Co., The Woodlands, Tex.
46. **Reiser, J., A. K. Koch, U. A. Ochsner, and A. Fiechter.** 1993. Genetics of surface-active compounds, p. 231–249. *In* N. Kosaric (ed.), Biosurfactants: production, properties, applications. Marcel Dekker Inc., New York.
47. **Reiser, J., and G. R. Stark.** 1983. Immunologic detection of specific proteins in cell extracts by fractionation in gels and transfer to paper. Methods Enzymol. **96**:205–215.
48. **Renart, J., J. Reiser, and G. R. Stark.** 1979. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. Proc. Natl. Acad. Sci. USA **76**:3116–3120.
49. **Rosenberg, E.** 1993. Exploiting microbial growth on hydrocarbons—new markets. Trends Biotechnol. **11**:419–424.
50. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
51. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166**:268–379.
52. **Schweizer, H. P.** 1991. Improved broad-host-range *lac*-based plasmid vectors for the isolation and characterization of protein fusions in *Pseudomonas aeruginosa*. Gene **103**:87–92.
53. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. λ ZAP: a bacteriophage lambda expression vector with *in vivo* excision properties. Nucleic Acids Res. **16**:7583–7600.
54. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
55. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
56. **Syldatk, C., and F. Wagner.** 1987. Production of biosurfactants, p. 89–120. *In* N. Kosaric, W. L. Cairns, and N. C. C. Gray (ed.), Biosurfactants and biotechnology. Marcel Dekker Inc., New York.
57. **Tabor, S., and C. C. Richardson.** 1987. DNA sequencing with a modified bacteriophage T7 polymerase. Proc. Natl. Acad. Sci. USA **84**:4767–4771.
58. **Van Dyke, M. I., S. L. Gulley, H. Lee, and J. T. Trevors.** 1993. Evaluation of microbial surfactants for recovery of hydrophobic pollutants from soil. J. Ind. Microbiol. **11**:163–170.
59. **Van Dyke, M. W., M. Sirito, and M. Sawadogo.** 1992. Single-step purification of bacterially expressed polypeptides containing an oligo-histidine domain. Gene **111**:99–104.
60. **Velikonja, J., and N. Kosaric.** 1993. Biosurfactants in food applications, p. 419–446. *In* N. Kosaric (ed.), Biosurfactants: production, properties, applications. Marcel Dekker Inc., New York.
61. **Wagner, F., U. Behrendt, H. Bock, A. Kretschmer, S. Lang, and C. Syldatk.** 1983. Production and chemical characterization of surfactants from *Rhodococcus erythropolis* and *Pseudomonas* sp. MUB grown on hydrocarbons, p. 55–60. *In* I. E. Zajic, D. G. Cooper, T. R. Jack, and N. Kosaric (ed.), Microbial enhanced oil recovery. Pennwell Books, Tulsa, Okla.
62. **Walter, M. V., A. Porteous, and R. J. Seidler.** 1987. Measuring genetic stability in bacteria of potential use in genetic engineering. Appl. Environ. Microbiol. **53**:105–109.
63. **West, S. E. H., and B. H. Iglewski.** 1988. Codon usage in *Pseudomonas aeruginosa*. Nucleic Acids Res. **16**:9323–9335.