JOURNAL OF BACTERIOLOGY, Nov. 2001, p. 6579–6589 Vol. 183, No. 22
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.22.6579–6589.2001
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Characterization of Phenotypic Changes in *Pseudomonas putida* in Response to Surface-Associated Growth

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Received 30 July 2001/Accepted 21 August 2001

The formation of complex bacterial communities known as biofilms begins with the interaction of planktonic cells with a surface. A switch between planktonic and sessile growth is believed to result in a phenotypic change in bacteria. In this study, a global analysis of physiological changes of the plant saprophyte *Pseudomonas putida* following 6 h of attachment to a silicone surface was carried out by analysis of protein profiles and by mRNA expression patterns. Two-dimensional (2-D) gel electrophoresis revealed 15 proteins that were up-regulated following bacterial adhesion and 30 proteins that were down-regulated. N-terminal sequence analyses of 11 of the down-regulated proteins identified a protein with homology to the ABC transporter, PotF; an outer membrane lipoprotein, NlpD; and five proteins that were homologous to proteins involved in amino acid metabolism. cDNA subtractive hybridization revealed 40 genes that were differentially expressed following initial attachment of *P. putida*. Twenty-eight of these genes had known homologs. As with the 2-D gel analysis, NlpD and genes involved in amino acid metabolism were identified by subtractive hybridization and found to be down-regulated following surface-associated growth. The gene for PotB was up-regulated, suggesting differential expression of ABC transporters following attachment to this surface. Other genes that showed differential regulation were structural components of flagella and type IV pili, as well as genes involved in polysaccharide biosynthesis. Immunoblot analysis of PilA and FliC confirmed the presence of flagella in planktonic cultures but not in 12- or 24-h biofilms. In contrast, PilA was observed in 12-h biofilms but not in planktonic culture. Recent evidence suggests that quorum sensing by bacterial homoserine lactones (HSLs) may play a regulatory role in biofilm development. To determine if similar protein profiles occurred during quorum sensing and during early biofilm formation, HSLs extracted from *P. putida* and pure C_{12}-HSL were added to 6-h planktonic cultures of *P. putida*, and cell extracts were analyzed by 2-D gel profiles. Differential expression of 16 proteins was observed following addition of HSLs. One protein, PotF, was found to be down-regulated by both surface-associated growth and by HSL addition. The other 15 proteins did not correspond to proteins differentially expressed by surface-associated growth. The results presented here demonstrate that *P. putida* undergoes a global change in gene expression following initial attachment to a surface. Quorum sensing may play a role in the initial attachment process, but other sensory processes must also be involved in these phenotypic changes.

In the vast majority of ecosystems, microbial cells grow in association with surfaces (9, 10, 11, 12). Surface-associated growth leads to the formation of a biofilm, a highly structured, sessile microbial community (30). The formation of a mature biofilm is believed to occur in a sequential process of (i) transport of microorganisms to a surface, (ii) initial microbial attachment, (iii) formation of microcolonies, and (iv) formation of mature biofilms (41, 65). Cellular components are required for the sequence of events leading to mature biofilm formation, and changes in gene expression likely lead to changes in these cellular components.

Of the processes leading to mature biofilm development, bacterial structural components for intial attachment have been best characterized, primarily through mutation analysis. Specific structural components shown to play a critical role in facilitating bacterial interaction with surfaces include flagella, pili, and adhesins. The primary function of flagella in biofilm formation is assumed to be in transport and in initial cell-to-surface interactions. The absence of flagella impaired *Pseudomonas fluorescens* and *Pseudomonas putida* in colonization of potato and wheat roots (18, 20) and reduced cellular adhesion of *Pseudomonas aeruginosa* to a polystyrene surface (49). Pili and pilus-associated adhesins have been shown to be important for the adherence to and colonization of surfaces. In *Escherichia coli*, attachment is reduced by mutations in the *csgA* gene, a biosynthetic curlin gene (22, 67), and in the type I pili biosynthesis gene *fimH*, which encodes the mannose-specific adhesin in *E. coli* (52). There is also evidence for adhesive properties of type IV pili of *P. aeruginosa*, since mutants were reduced in the ability to form microcolonies when absent (49). Mutations in *ica*, the gene for the polysaccharide intercellular adhesin of *Staphylococcus epidermidis*, in *atlE*, the gene for autolysin of *Staphylococcus aureus* (34, 40, 57), and in the gene for the mannose-sensitive hemagglutinin pilus of *Vibrio cholerae* El Tor (68) all reduced adhesion to surfaces.

Membrane proteins may also influence bacterial attachment processes. Mutations in surface and membrane proteins, including a calcium-binding protein, a hemolysin, a peptide transporter, and a potential glutathione-regulated K^+ efflux pump caused defects in attachment of *P. putida* to corn (25). The requirement for ABC transport systems in attachment and virulence was also demonstrated in *Agrobacterium tumefaciens*. The deletion of genes encoding components of the polyamine

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ABC transporter *potB*, *potH*, *potC*, and *potI* abolished attachment of *A. tumefaciens* to carrot suspension culture cells, and the resulting deletion mutants were avirulent (42). Bacterial extracellular polysaccharides may also influence attachment and initial biofilm development, since these factors contribute to cell surface charge, which affects electrostatic interactions between bacteria and substratum (66). Adhesiveness of *Pseudomonas* species is related to the presence and composition of lipopolysaccharides (71). Substantially reduced attachment to biotic and abiotic surfaces was observed in O-polysaccharide-deficient *Pseudomonas* spp. (17, 19) and in *E. coli* strains with mutations in the lipopolysaccharide core biosynthesis genes *rfaG*, *rfaP*, and *galU* (19, 31, 56). The extracellular polysaccharide alginate was required for formation of thick, three-dimensional *P. aeruginosa* biofilms and was shown to be the intercellular material of *P. aeruginosa* microcolonies (45).

Less is known about the cascade of events following adhesion than about the adhesion process. Attachment to surfaces is thought to initiate a cascade of changes in the bacterial cells. Examples of changes in gene expression following bacterial adhesion include surface-induced gene activation of *P. aeruginosa algC*, a gene involved in lipopolysaccharide core biosynthesis and in the biosynthesis of the exopolysaccharide alginate (15, 16). In *E. coli*, up-regulation after attachment was observed for OmpC, the *proU* operon, colanic acid exopolysaccharide production, tripeptidase T, and the nickel high-affinity transport system (*nikA*) (53). Changes in gene expression that correlate with attachment to surfaces have also been described for antibiotic resistance, including β-lactamase activity in *P. aeruginosa* (4, 32), and for antibiotic production such as phenazine synthesis in *Pseudomonas aureofaciens* (72).

The expression of phenazines as well as of numerous other virulence factors is under the control of quorum sensing (26, 70). Recent studies have linked quorum sensing and biofilm formation. Developmental processes such as maturation of biofilms and differentiation into microcolonies were shown to be dependent on the signal molecule *N*-3-(oxooctanoyl)-L-homoserine lactone ($3OC_{12}$-HSL). This finding led to speculation that cell-to-cell signaling induced by the high density of bacteria within biofilms may play a role in the establishment of a biofilm-specific physiological state (14).

In this study, to further characterize the sequential process involved in biofilm development, we focused on the phenotypic changes that occur in the initial phases of biofilm formation soon after bacterial adhesion. The soil bacterium *P. putida* was chosen for this study, since this bacterium colonizes the surface of plant roots and promotes plant growth. To begin these investigations, we used two approaches: (i) proteomic analysis of whole-cell extracts prior to and following bacterial adhesion and (ii) cDNA subtractive hybridization of mRNA prior to and following adhesion. The proteomic approach was also used to address the role of cell signaling by HSLs in biofilm development soon after bacterial adhesion. These studies indicate that *P. putida* undergoes a variety of structural and metabolic changes following initial adhesion to a surface and that cell-cell signaling may be only partially responsible for these regulatory changes in the metabolic and structural components.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The microorganism used in this study was a plant growth promoting *P. putida* (ATCC 39168). *P. putida* was grown at room temperature in chemostats (300 ml; flow rate, 1.7 ml/min) in minimal medium (2.56 g of Na_2HPO_4, 2.08 g of KH_2PO_4, 1.0 g of NH_4Cl, 0.132 g of $CaCl_2 \cdot 2H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 mg of $CuSO_4 \cdot 5H_2O$, 0.1 mg of $ZnSO_4 \cdot H_2O$, 0.1 mg of $FeSO_4 \cdot 7H_2O$, and 0.004 mg of $MnCl_2 \cdot 4H_2O$ per liter [pH 7.0]). Glutamic acid (130 mg/liter) was used as the sole carbon source. The residence time of *P. putida* cells in the chemostat was 170 min in comparison to the doubling time of 120 min in suspension. For the acylated-HSL (AHL) add-back assay, *P. putida* was grown in chemostats in minimal medium supplemented with 10 μM $3OC_{12}$-HSL. $3OC_{12}$-HSL was chosen because of its involvement in maturation of biofilms (14). *A. tumefaciens* A136 (Ti-negative) (pCF218) (pCF372) and *A. tumefaciens* KYC6 (27) were used as indicator strains for the detection of AHLs. *A. tumefaciens* A136 and *A. tumefaciens* KYC6 were kindly provided by C. Fuqua. The genetic element pCF218 codes for the Tra protein, an AHL-responsive transcription factor that recognizes $3OC_{12}$-HSL and a wide range of AHLs with various acyl chains (27, 28). The Tra-regulated *traI-lacZ* reporter is carried on the plasmid pCF372. *A. tumefaciens* KYC6 was used as an endogenous AHL overproducer. *A. tumefaciens* A136 and *A. tumefaciens* KYC6 were grown at 30°C on ATGN minimal medium [*A. tumefaciens* minimal salts medium with 15 mM $(NH_4)_2SO_4$ and 0.5% glucose] supplemented with the appropriate antibiotics as described by Fuqua and Winans (27). The PilA mutant *P. aeruginosa* PA416 was used for immunoblot analysis and grown planktonically in Luria-Bertani medium.

Reagents. Immobiline Dry-Strips, dithiothreitol, Pharmalyte 3-10, and Coomassie brilliant blue R350 were purchased from Amersham Pharmacia (Piscataway, N.J.). Urea, thiourea, sodium dodecyl sulfate (SDS), Tris base, glycine, biuret reagents, and acryl/bisacrylamide were from Sigma (St. Louis, Mo.). Phenylmethylsulfonyl fluoride was from Boehringer Mannheim (Indianapolis, Ind.), iodoacetamide from Acros Organics (Somerville, N.J.), and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was from Pierce (Rockford, Ill.). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was purchased from Polyscience (Warrington, Pa.).

Biofilm growth following initial attachment. The interior surfaces of silicone tubing (Masterflex) were used to cultivate biofilms. Each silicone tube was 1 m in length (size 16, resulting volume of 7 ml). Cultures of *P. putida* were grown in chemostats prior to the inoculation of silicone tubing by syringe injection. Four milliliters of chemostat-grown culture of *P. putida* was injected into the tubing and allowed to attach for 30 min before the flow of minimal medium (0.4 ml/min) was initiated. The residence time in the tubing was 17.5 min, less than the doubling time of *P. putida* in suspension, allowing only attached organisms to be retained within the tubing. After various times up to 24 h, attached cells were removed from the interior surface by squeezing the tubes, followed by extrusion of the cell material from the lumen. The resulting cell suspensions were harvested by centrifugation at 12,000 × *g* for 10 min at 4°C. Experiments for each time point were repeated at least five times. Medium effluents from tubing were collected in 30-min intervals over a period of 12 h after initial attachment and were immediately placed on ice. Collected effluents were centrifuged at 12,000 × *g* for 10 min at 4°C, and the cell-free supernatant was stored at −20°C. The effluents (up to 250 ml) were then processed for AHL extraction and analyzed for the presence of AHLs as described below. AHL experiments were repeated three times.

Preparation of crude protein extract. Chemostat-grown *P. putida* cells were harvested by centrifugation for 10 min at 16,300 × *g* at 4°C. The pellets were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), containing 0.3 mg of phenylmethylsulfonyl fluoride/ml, and cells were disrupted by sonication (6 times for 10 s, 4 W, 4°C) (Cole Parmer Instruments Co., Vernon Hills, Ill.). Cell debris and unbroken cells were removed by centrifugation (30,600 × *g*, 30 min, 4°C). The crude protein extract was either stored at −20°C or was immediately processed for electrophoresis. Total protein concentration was determined by the modified version (51) of the method of Lowry et al. using reagents from Sigma. Bovine serum albumin was used as the standard.

2-D gel electrophoresis. Two-dimensional (2-D) gel electrophoresis was conducted according to the principles of O'Farrell (47) as outlined by Görg et al. (33). Isoelectric focusing (IEF) was performed using individual Immobiline Dry-Strips (18 cm, pH 3 to 10 nonlinear; Pharmacia) using a Multiphor II from Pharmacia. Crude protein extracts, (500 μg) were solubilized in 450 μl of a solution containing urea, thiourea, dithiothreitol, CHAPS, and Pharmalyte 3-10. Samples were applied to the strips by in-gel rehydration. IEF was performed initially at low voltage (500 V), and then the voltage was increased to 3,500 V at a constant temperature of 20°C. IEF was continued at 3,500 V for a total of 35

kVh. The Immobiline Dry-Strips were equilibrated (33) and were subsequently applied to SDS gels. For the resolution of *P. putida* crude protein extracts in the second dimension, the 20- by 20-cm 2-D gel system from Bio-Rad was used. Crude protein extracts were separated on 11% resolving gels at 10°C. Gels were stained with Coomassie brilliant blue R350. 2-D gel analysis was repeated at least three times for each growth condition.

Immunoblot analyses. Planktonic and sessile cultures were cultivated as described above. Cells were harvested at various time intervals for up to 7 days. Whole cells were lysed with SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (200 mM Tris, 1% SDS, 80 μM EDTA, and 26 mM dithiothreitol [pH 8.0]). Proteins were separated on 10% resolving and 4% stacking gels using SDS electrophoresis according to Laemmli (38). Following SDS-PAGE, proteins were electroblotted onto nitrocellulose membranes (3). The membranes were probed with polyclonal antibodies for FliC (b-type flagella) that were kindly provided by D. Wozniak or monoclonal antibodies for the type IV pilin protein PilA that were kindly provided by W. Shi. Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase was used as the secondary antibody. Antibody binding was detected by colorimetric analysis (3).

AHL extraction, cross-feeding assay for AHL detection, and AHL separation by HPLC. AHL preparations were isolated from cell-free supernatants and were extracted with acidified ethyl acetate as described by Shaw et al. (59). To assay for the presence of AHL in culture supernatants and effluents of silicone tubing from biofilm experiments, AHLs were extracted and assayed in the cross-feeding assay using *A. tumefaciens* A136 as a reporter strain, as described by Stickler et al. (62). Briefly, *A. tumefaciens* A136 was inoculated onto ATGN agar containing X-Gal (40 μg/liter), and culture supernatant extracts were spotted onto the medium. β-Galactosidase activity of the reporter strains was indicative of the presence of AHLs. Positive and negative controls consisted of culturing the reporter strain with *A. tumefaciens* KYC6 (AHL overproducer) and with *A. tumefaciens* A136 (which does not produce AHL). The separation and identification of signaling molecules synthesized by *P. putida* were performed by high-performance liquid chromatography (HPLC) (58). Briefly, culture supernatants were collected, extracted with ethyl acetate as described by Shaw et al. (59), and separated on a C_{18} reverse-phase column (catalog no. 504971 ambient; Supelco) as described by Schaefer and coworkers (58). The collected 1-ml fractions were assayed in the cross-feeding assay using *A. tumefaciens* A136 as a reporter strain, as described by Stickler et al. (62). The synthetic AHL $3OC_{12}$-HSL and the ethyl-acetate-extractable *P. aeruginosa* AHLs (C_6-HSL, C_8-HSL, C_{10}-HSL, and $3OC_{12}$-HSL) were used as standards.

N-terminal sequencing. For the determination of N-terminal amino acid sequences, crude protein extracts were separated by 2-D electrophoresis and blotted onto an Immobilon-P membrane (Millipore, Bedford, Mass.), in blotting buffer (25 mM Tris, 0.01% SDS, 192 mM glycine, and 20% methanol). The membrane was stained for 5 min with Coomassie brilliant blue R250 (0.1% in 50% methanol) and was destained for 2 min in 50% methanol. The areas containing proteins of interest were excised. N-terminal sequence determination was performed by the Protein Chemistry Laboratory at the University of Texas Medical Branch, Galveston, Tex., by Edman degradation (23). Proteins were identified using the BLAST program (1) of the annotated *P. aeruginosa* genome (www.pseudomonas.com) and the National Center for Biotechnology Information BLAST website for "short nearly exact matches."

In vitro polyadenylation and subtractive hybridization. Planktonic and sessile *P. putida* cells were cultivated as described above. For the preparation of *P. putida* mRNA, a method based on selective in vitro polyadenylation was used (69). The modification is based on the in vitro polyadenylation of bacterial RNA. Polyadenylation was carried out using yeast poly(A) polymerase I (U.S. Biochemicals, Cleveland, Ohio). After polyadenylation, the RNA was isolated using the phenol-guanidinium thiocyanate-based Tri Reagent (LS system; Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's protocol. Subtractive hybridization was carried out as described by Diatchenko et al. (21) and was repeated twice. The gene for α-ketoglutarate dehydrogenase, *kgdA*, was used as internal control to determine the efficiency of subtractive hybridization. The polyadenylation of bacterial mRNA allowed the use of commercially available PCR-Select cDNA subtractive hybridization kits (Clontech Laboratories, Palo Alto, Calif.) which were designed for eukaryotic mRNA and were based on the presence of a poly(A) tail. The selectively enriched cDNAs were cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, Calif.). The inserts were sequenced using the M13 forward and M13 reverse standard primers and the Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). Typically, DNA inserts of 100 to 450 bp in length were obtained. The sequences were identified by searching for homologous sequences in the unfinished *P. putida* KT2410 Genome Project website (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi), and the *P. aeruginosa* Genome Project website (http://www.pseudomonas.com), using the BLASTN and BLASTX programs (1). The number of surface-regulated genes that belong to one operon was defined by analyzing the position of rho-independent terminators and the organization of the operon. For the identification of the position of rho-independent terminators and the operon structure, the http://pseudomonas.bit.uq.edu.au and http://www.pseudomonas.com websites were used.

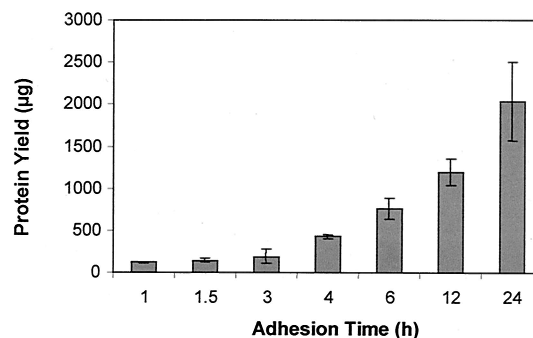

FIG. 1. Protein yield from tubing after different attachment times. For each attachment time point, the cell suspensions of four silicone tubes were combined and harvested by centrifugation.

RESULTS

***P. putida* forms biofilms on silicone surfaces.** To determine if *P. putida* was able to attach to and grow on silicone surfaces, planktonic *P. putida* cells were exposed to the interior surfaces of silicone tubing for 30 min, followed by the flow of fresh minimal medium. At various time points, the attached cells were harvested from the interior surface of silicone tubing and the protein yield was determined. Attached cells apparently experience a lag phase in growth as indicated by a period of 2 to 3 h of minimal increase in protein (Fig. 1). Slower growth of *P. putida* cells upon attachment was confirmed microscopically by following attached cells over a period of 6 h. Within this time only two cell divisions could be observed (not shown). After this initial lag, the protein yield increased, indicating bacterial growth on the surface of the tubing. The turbidity of the harvested cell suspension also increased over time up to an optical density at 600 nm of ~0.6 at 24 h (not shown).

Proteome analysis reveals 45 differences in the protein profiles of planktonic cells and sessile cells. Whole crude protein extracts from planktonic chemostat-grown *P. putida* were analyzed by 2-D gel electrophoresis. A representative example of the 2-D gels with more than 1,000 distinct protein spots is shown in Fig. 2. The 2-D gels of crude protein extracts comprise protein patterns with a high density of spots in the neutral range, with lower densities in the acidic and basic pH range. Crude protein extracts obtained from *P. putida* grown in a chemostat or attached to silicone tubing for 4, 6, 12, or 24 h were analyzed by 2-D gel electrophoresis. Alteration of protein patterns of attached *P. putida* in comparison to those of planktonic cells was visible as early as 4 h after initial attachment. 2-D gels were repeated for each growth condition independently at least three times to confirm the reproducibility of the protein pattern under planktonic and attached growth conditions. Only differences in protein spots that were reproduced three times are described here. Protein patterns obtained from attached cells after 4 and 6 h of attachment time were similar. In contrast, additional changes in the protein patterns were

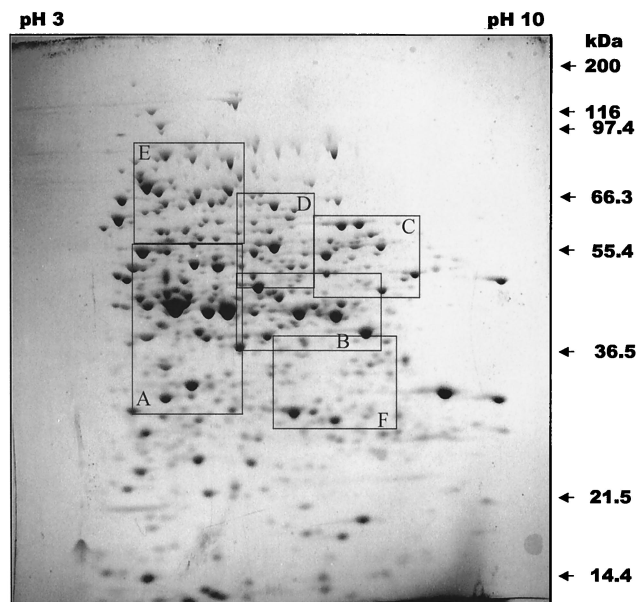

FIG. 2. 2-D images of crude protein extracts of planktonic *P. putida* grown in a chemostat. The crude protein extracts (500 μg) were extracted and separated on nonlinear Immobiline Dry-Strips (pH 3 to 10), followed by SDS–11% polyacrylamide gels. The gels were stained with Coomassie brilliant blue. The boxes A to F indicate areas that are enlarged in Fig. 3 and 5.

observed after 12 h of biofilm growth (not shown). In this study, we were interested in changes in protein patterns soon after initial adhesion; therefore, differences in planktonic cultures and cells cultivated for 6 h on silicone surfaces were chosen for additional study.

Comparison of protein patterns of planktonic and 6-h *P. putida* biofilms demonstrated increased concentration of 15 proteins and decreased concentration of 30 proteins (Fig. 3). A detailed comparative view is shown in Fig. 3 presenting enlarged sections from the 2-D image in Fig. 2. Most of the differentially expressed proteins are located in the neutral-to-basic pH range and showed an average molecular mass of 30 to 60 kDa. Several proteins were easily detected in attached cells that were only weakly detected in protein patterns derived from planktonic cells (Fig. 3, spots C1 to C6). Similarly, protein spots indicated by A1 to A11 were produced at higher levels in planktonic cells than in attached cells. Protein spots that were absent in attached cells but present in planktonic cells are indicated as B1 to B9, and the spot D1 was present in attached cells but not in planktonic culture.

Protein N-terminal sequence analysis indicates reduced levels of membrane proteins, NlpD, PotF, and amino acid biosynthetic proteins. Using sequencing by Edman degradation, we were able to obtain N-terminal sequences for 10 of the 45 differentially expressed proteins (Table 1). The proteins for which N-terminal sequences were obtained were down-regulated following adhesion to and growth on the silicone surface (as indicated by boxes in Fig. 3). Proteins were then identified by comparing the N-terminal amino acid sequence with the Genome Project website of *P. aeruginosa* and the unfinished genome sequence of *P. putida* (http://www.tigr.org). Sequence analysis indicated that protein A8 had homology to the outer membrane lipoprotein NlpD of *P. aeruginosa*. This protein is thought to have cell wall lytic function (39). Protein A6 had homology to PotF1 of *P. fluorescens* and PotF2 of *P. aeruginosa*. In *P. fluorescens*, PotF1 functions as a periplasmic component of the putrescine transport system. A second protein,

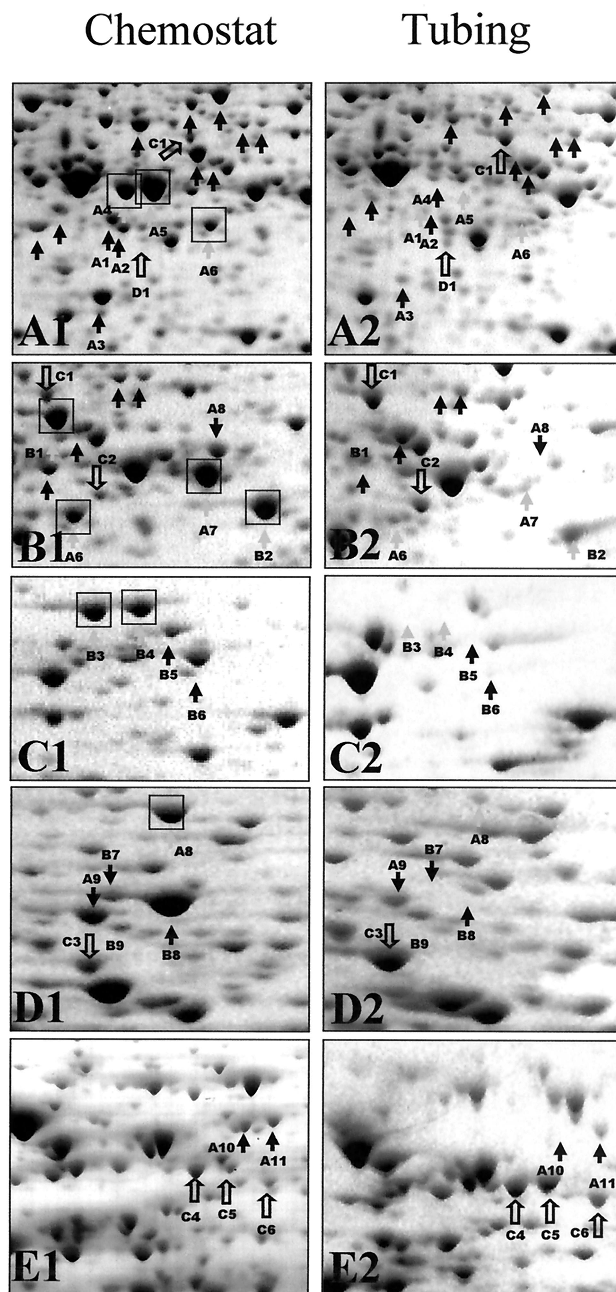

FIG. 3. Enlarged partial 2-D gels showing crude protein extract of planktonic *P. putida* grown in a chemostat (A1 to E1) and after a period of 6 h of attachment time (A2 to E2). The sections A1 to E1 show an enlarged view of the 2-D image in Fig. 2. The sections A2 to E2 are the corresponding sections in the 2-D gel of crude protein extracts obtained under attached growth conditions. Open arrows indicate protein spots, which are up-regulated in attached cells, while solid arrows mark those protein spots that are up-regulated in planktonic cells.

TABLE 1. Identification and function of selected 2-D gel protein spots that were down-regulated after 6 h of attachment time[a]

Spot	N-terminal sequence	Locus	Protein	Protein description	Function
A8	MLIVTKNPPVVGHDQ	PA3623	NIpD	Outer membrane lipoprotein	Cell wall
A6	DDKVLHVVN-D–A	PA0300	PotF2	ABC transporter	Polyamine transport
B9	DVKIGVAGPMTDA	PA4913	NI[b]	ABC transporter	Amino acid metabolism
B2	KEAETVQKLANVVIL	PA1337	AnsB	Glutaminase asparaginase	Amino acid metabolism
A4	SAEKQK-GVHSEAGK	PA5171	ArcA	Arginine deiminase	Amino acid metabolism
A7	AFNIHNRNLL	PA5172	ArcB	Ornithine carbamoyltransferase	Amino acid metabolism
B1	MFSRDLTIAK-DA-L	PA4602	GlyA3	Serine-hydroxymethyltransferase	Amino acid metabolism
B4	-PATK-P	No match			
B3	PATA-PAGKN	No match			
A5	AD-IKI-GAQ-QD	No match			

[a] The protein spots were N-terminally sequenced, and the proteins were identified by comparing the N-terminal amino acid sequence to that found on the *P. aeruginosa* website using the BLASTX program (1). The spot numbers correlate with the numbers indicated in Fig. 3. The locus designates the gene number in the genome of *P. aeruginosa*.

[b] NI, not identified.

B9, putatively identified as a transport protein, was found to be down-regulated following adhesion of *P. putida*. B9 had homology to PA4913, a probable binding protein component of an ABC transporter for branched-chain amino acid. Other proteins showing reduced concentration following 6 h of biofilm growth were B2, A4, A7, and B1. Sequences of these proteins were homologous to AnsB, ArcA, ArcB, and GlyA3 of *P. aeruginosa*. These proteins are likely involved in amino acid metabolism. Interestingly, both planktonic and biofilm cultures were cultivated with glutamate as the sole carbon source. Therefore, changes in amino acid metabolic proteins suggest that in addition to alterations in structural components of cells following initial adhesion to a surface, bacteria may also undergo metabolic changes. An N-terminal sequence was obtained for three additional proteins that showed reduced concentration following attachment, B4, B3, and A5. At this time, we are unable to find homologs to these proteins in the *P. aeruginosa* genome database. These proteins may represent proteins that are unique to *P. putida*.

Subtractive hybridization reveals at least 40 differences in mRNAs of planktonic and 6-h biofilms. Due to the low concentration of the remaining protein spots that showed different concentrations in attached and planktonic cells, we were unable to obtain N-terminal sequence information for these proteins. Matrix-assisted laser desorption ionization–time of flight (mass spectrometry) has a greater limit of detection. However, the lack of a completed genome sequence of *P. putida* makes further identification of differential protein spots via matrix-assisted laser desorption ionization–time of flight (mass spectrometry) difficult at this time. Therefore, in order to obtain additional information on phenotypic changes following attachment to a surface, we utilized an alternative strategy of subtractive hybridization. This technique allows the selective enrichment of cDNA synthesized from mRNA found under one growth condition (for example, planktonic growth) but not under another condition (for example, sessile growth). After hybridization of cDNAs that were obtained under both conditions, unpaired, single-stranded cDNAs were PCR amplified, cloned, and sequenced. Protein homologs to cDNA sequences were identified in unfinished *P. putida* KT2410 Genome Project and the *P. aeruginosa* Genome Project. The results from this comparison are given in Table 2.

Genes showing differential regulation following 6 h of biofilm growth fell into four general classes (Table 2). Class I included genes that encode factors for metabolic processes, such as amino acid metabolism, carbon catabolism, and cofactor biosynthesis. As was the case for the proteome analysis, the genes for amino acid metabolism, as well as the other metabolic genes, had reduced expression following 6 h of biofilm growth. Class II contained membrane proteins primarily involved in molecular transport. Included in this class of proteins was the outer membrane lipoprotein, *nlpD*, which was also identified in the proteome analysis. Both assays showed a decreased expression of *nlpD* following bacterial adhesion. *potB*, a gene encoding the membrane-spanning protein of the ABC transport system for polyamine, was found to be up-regulated following adhesion. PotB was previously shown to be required for adhesion and virulence of *A. tumefaciens* to carrot cells (42). Interestingly, this contrasts with the down-regulation of PotF2 as demonstrated by the 2-D gel analysis. PotB and PotF2 are encoded on separate biosynthetic operons. Other membrane proteins that were up-regulated following adhesion were *mexB* and *xcpS* (Table 2). Class III included proteins involved in polysaccharide biosynthesis. Genes involved in lipopolysaccharide biosynthesis, *lpxD* (61) and *wbpG* (55), were found to be up-regulated following attachment. *mucC*, a putative negative regulator of alginate biosynthesis, was also up-regulated. *mucC* is contained on an operon of alginate regulatory genes, *algT(U)*>*mucA*>*mucB*>*mucC*>*mucD*. Class IV included proteins involved in adhesion and motility. Genes involved in pilus biosynthesis, *pilC*, *pilR*, and *pilK*, were found to be up-regulated following adhesion, whereas genes involved in flagellar biosynthesis, *fleN* and *flgG*, were down-regulated. NrqB, which may be involved in energetics of flagellar rotation, was also down-regulated following adhesion. Other genes found to be down-regulated following adhesion were *recB* and *ksgA*. Potential virulence factors *chiC* and *ampC* were up-regulated. Twelve genes, five surface repressed and seven surface induced, could not be identified by sequence homology (data not shown).

Immunoblot analysis confirms differential expression of pili and flagella following bacterial adhesion. Subtractive hybridization indicated differential expression of pili and flagella following adhesion of *P. putida* to a surface, suggesting a surface-regulated switch from flagellum-based motility to swarming or twitching motility (Table 2). Immunoblot analysis was used to further characterize this switch. Bacteria grown in biofilms for 12 h, 1 day, 3 days, and 7 days were analyzed by SDS-PAGE

TABLE 2. Identification and function of genes which are differentially expressed in *P. putida* 6 h after initial attachment to the inner surface of tubing[a]

Class and function	Gene	Expression	Locus
Class I			
Carbon catabolism/amino acid metabolism cofactor metabolism			
Two-component response	*gltR*	−	PA3192
Ribokinase	*rbsK*	−	PA1950
Probable asparagine synthetase	*asnB*	−	PA2084
Probable acyl-coenzyme A dehydrogenase	NI	−	PA2015
Probable aldehyde dehydrogenase	*adhA*	−	PA2217
Leucyl-tRNA synthase	*leuS*	−	PA3987
Thiamine phosphate pyrophosphorylase, thiamine	*thiE*	−	PA3976
Hypothetical protein, ubiquinone biosynthesis protein	*aarF*	−	PA5065
Class II			
Membrane proteins/transport			
Outer membrane lipoprotein	*nlpD*	+	PA3623
ABC transporter	*potB*	+	PA3608
Resistance/nodulation/cell division multidrug efflux pump	*mex*	+	PA0425
Probable K^+ efflux transporter	*ybaL*	+	PA5518
General secretion pathway protein F	*xcpS*	+	PA3102
Class III			
Polysaccharides/lipopolysaccharide biosynthesis			
Negative regulator for alginate biosynthesis	*mucC*	+	PA0765
Putative capsule polysaccharide export protein precursor (*Klebsiella pneumoniae* partial YC04 gene)	NI	+	NI
UDP-3-*O*-[hydroxylauroyl] glucosamine *N*-acyltransferase	*lpxD*	+	PA3646
Lipopolysaccharide biosynthesis gene	*wbpG*	+	PA3150
Class IV			
Motility			
Flagellar synthesis regulator	*fleN*	−	PA1454
Flagellar basal body rod protein	*flgG*	−	PA1082
Na^+-translocating NADH:ubiquinone oxidoreductase Nrq2	*nqrB*	−	PA2998
Two-component response regulator	*pilR*	+	PA4547
Type IV fimbrial biosynthesis gene	*pilC*	+	PA4527
Chemotactic methyltransferase CheR homolog	*pilK*	+	PA0412
DNA replication and rRNA maturation			
Exoribonuclease V beta chain	*recB*	−	PA4284
rRNA (adenine N6, N6)-dimethyltransferase	*ksgA*	−	PA0592
Antibiotic resistance/virulence factors			
Chitinase	*chiC*	+	PA2300
β-Lactamase	*ampC*	+	PA4410
Streptomycin 3′-phosphotransferase	*str*	+	PA1858

[a] Subtracted cDNA libraries were generated using subtractive hybridization and the resulting DNA sequences representing differentially expressed genes were identified by sequence comparison. The identification was carried out using the BLASTN and BLASTX program (1). The gene names correspond to the gene names given in the *P. aeruginosa* Genome Project website. NI, not identified. −, down-regulated in attached *P. putida* cells; +, up-regulated in attached *P. putida* cells.

and probed with polyclonal antibodies for b-type flagella (FliC) and with monoclonal antibodies for type IV pili. Immunoblot analysis revealed the presence of FliC in planktonic cultures but the absence of flagella in 12-h and 1-day biofilms (Fig. 4A). Interestingly, after 3 days of biofilm development b-type flagella were again detectable (Fig. 4A), suggesting that flagella may be required for biofilm dispersion. Immunoblot analysis also confirmed surface-induced expression of type IV pili following bacterial adhesion (Fig. 4B). PilA was not observed in planktonic culture (although a larger cross-reactive band was observed). However, PilA was detected throughout the course of 7 days of biofilm growth.

Production of AHLs by *P. putida*. Recent evidence suggests a role for cell-to-cell communication as a signaling mechanism in biofilm development (14). In particular, a LasI mutant of *P. aeruginosa*, incapable of C_{12}-HSL production, formed flat and undifferentiated biofilms, whereas addition of $3OC_{12}$-HSL to this mutant strain restored the differentiated biofilms, similar to the wild-type strain. In this study, we examined whether *P. putida* produced AHLs during biofilm growth and whether these AHLs played a role in the early biofilm development of *P. putida*. At least four AHLs were identified in this strain of *P. putida*. The retention times determined by HPLC for the four AHLs were identical to those for the AHLs of *P. aeruginosa* (data not shown) and the AHLs were tentatively identified as C_6-HSL, C_8-HSL, C_{10}-HSL, and $3OC_{12}$-HSL. These results confirm the results of studies by Elasri et al. (24) and Kojick et al. (36).

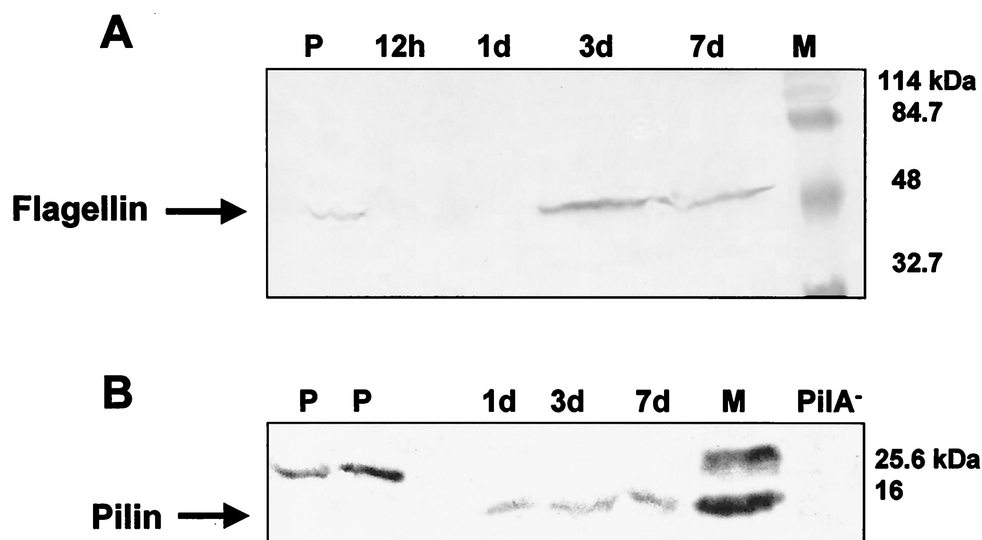

FIG. 4. Immunoblot of b-type flagella (A) and type IV pili (B) of whole *P. putida* cells grown in minimal medium in a chemostat or attached to silicone surface during biofilm development. Whole cells were analyzed by SDS-PAGE, and the proteins were electroblotted onto nitrocellulose membranes (3). The membranes were probed with polyclonal b-type flagella antibodies (A) or monoclonal type IV pilus antibodies (B). Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase was used as the secondary antibody. Antibody binding was detected by colorimetric analysis (3). M, marker; PilA$^-$, type-IV-pilus-deficient *P. aeruginosa* PA416 (49); P, planktonic, chemostat-grown *P. putida* cells; 12 h and 1 d, attached *P. putida* cells after 12 h and 1 day of attachment time, respectively.

The *A. tumefaciens* bioassay was used to detect AHL production in medium effluents from *P. putida* biofilms. No evidence of AHL activity was observed with filter-sterilized effluent collected between 0 and 7 h of initial biofilm formation or with these effluents that were concentrated 2,500-fold. A weak positive reaction was detected in effluents that were collected from 9 to 12.5 h, and a positive response was observed for 7- to 12.5-h effluents concentrated 2,500-fold. The results suggest that, during the initial biofilm development stage (i.e., 6 h following attachment), AHL production is below detection limits, likely due to the low cell densities during this time period.

AHL addition results in 16 differences in the protein profiles of 6-h planktonic cells. To determine if the changes in protein profiles observed following attachment to the silicone surfaces was due to cell signaling by AHLs, chemostat cultures of *P. putida* were incubated with AHLs extracted from *P. putida* supernatants or with synthetic 3OC$_{12}$-HSL. Protein extracts from these strains were then analyzed by 2-D gels and were compared to protein profiles from planktonic cultures without AHL addition and to profiles from 6-h biofilm cultures. Sections of these 2-D gels corresponding to those in Fig. 3 are shown in Fig. 5. The addition of 3OC$_{12}$-HSL to chemostat cultures caused the alteration of at least 16 proteins in the planktonic cells, including nine proteins that had increased concentration due to AHL addition and seven proteins that had decreased concentration (Fig. 5). Similar results were obtained when *P. putida* AHL extracts were added to the cultures (not shown). One protein spot (Fig. 5, spot A6, identified as PotF2) had reduced concentration both in the experiments with AHL addition and in the 6-h biofilm experiments. The remaining 15 spots, although yet to be identified, did not correspond to proteins that were differentially expressed during the 6-h biofilm experiment. Therefore, in the case of early biofilm development, changes in protein patterns and gene expression patterns must be a result of signaling other than cell signaling by AHLs.

DISCUSSION

The mechanisms of bacterial adhesion to surfaces have become increasingly well characterized (4, 18, 19, 20, 22, 25, 31, 32, 34, 40, 42, 45, 49, 50, 52, 56, 57, 67, 68, 71, 72). However, little is known regarding the events following bacterial adhesion and during biofilm development. It is thought that bacteria undergo a variety of phenotypic changes during biofilm development (14, 53). In the present work, we used proteomic analysis and subtractive cDNA libraries to characterize physiological changes of the bacterium *P. putida* during the initial phase of biofilm growth. The results indicated that the bacteria underwent a variety of metabolic changes in the first 6 h of biofilm growth. These changes included differential expression of proteins involved in amino acid metabolism, membrane proteins involved in transport process, and proteins involved in the production of extracellular polymers and organelles.

Some of the proteins and genes identified here have not been previously correlated with biofilm formation. For example, genes and gene products that represented evidence for changes in carbon and energy metabolism, cofactor biosynthesis, and slower growth were detected using both the proteomic and subtractive hybridization approaches. Genes and proteins involved in amino acid metabolism, including AsnB, ArcA, ArcB, and GlyA3, were down-regulated following initial attachment. Both chemostat and biofilm cultures were grown in minimal medium with glutamate as the sole carbon source. The results suggest that surface attachment and biofilm formation may not directly regulate some of the genes that we identified but may reflect a sequential process of establishing a

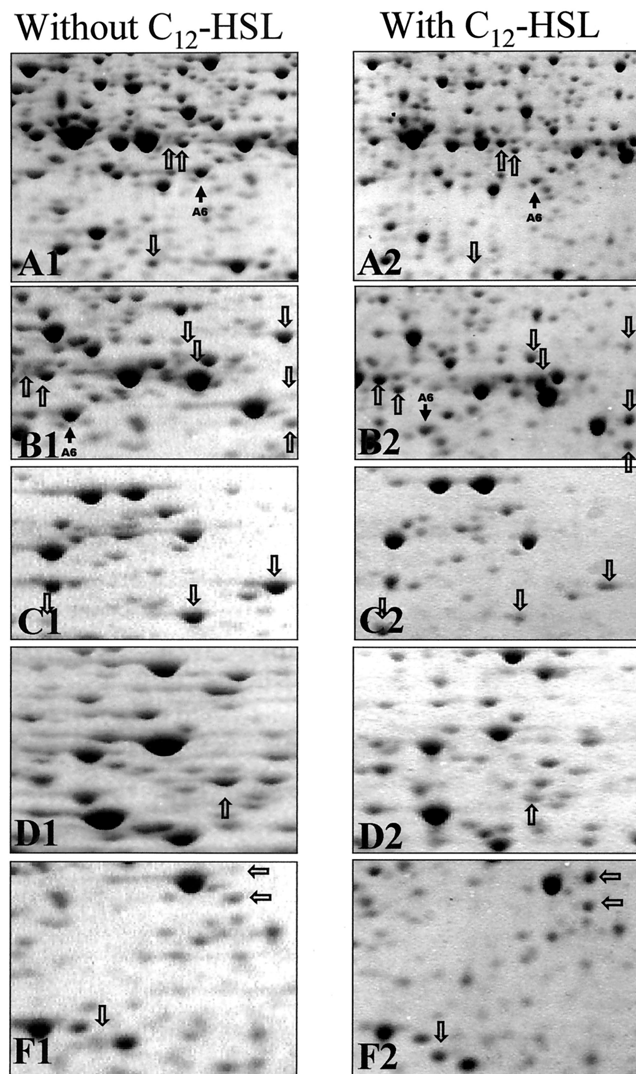

FIG. 5. Enlarged partial 2-D gels of crude protein extracts of *P. putida* in the absence (A1 to F1) and presence (A2 to F2) of the $3OC_{12}$-HSL signal molecule. The sections A1 to F1 and A2 to F2 correspond to the boxes A to F shown in Fig. 2. Arrows indicate differences in the 2-D protein pattern of chemostat-grown cells in the absence and presence (10 μM) of the C_{12}-HSL signal molecule.

population at a surface. Adaptation to growth on a surface may occur in a variety of organisms, since it appears that multiple pathways control biofilm formation and function under different growth conditions. For example, the phenotype of an attachment-defective *A. tumefaciens* strain grown in minimal medium could be suppressed by growth in conditioned medium (42). Similar results were obtained for a biofilm-defective phenotype of a subset of surface-attachment-deficient *P. fluorescens* mutants grown in glucose medium plus Casamino Acids. Attachment of this adhesion-deficient organism could be restored by growth on citrate or glutamate or in the presence of high iron concentrations (50). The medium composition that promotes attachment—and the subsets of genes required under each environmental condition—may simulate various niches that are normally colonized by this organism (50). In another study, Crc was suggested to be part of a signal transduction pathway that relays signals such as carbon availability and thereby regulates the transition from planktonic to biofilm growth (48). Crc plays a global role in carbon metabolism (catabolite repression control) and is also involved in twitching motility and in regulation of genes required for the synthesis of type IV pili (48). Slower growth of cells that initially colonize surfaces is consistent with earlier reports about *P. aeruginosa* showing that primary cells at a surface experience a lag phase in their growth (54). Among the other surface-repressed genes that indicated slower growth are *ksgA* and *recB*, involved in rRNA maturation and DNA replication, modification, and repair.

Membrane proteins have been reported to have a substantial influence on attachment and may also play a role in early biofilm development. The outer membrane lipoprotein NlpD was identified as down-regulated by both proteome analysis and by subtractive hybridization. NlpD is believed to have cell wall lytic function, since its C-terminal amino acid sequence shows homology to lysostaphin, an extracellular cell wall-degrading enzyme (39). Overproduction of NlpD in *E. coli* resulted in morphological aberrations associated with some serious defects in cell wall structure and the formation of bulges and eventually in cell lysis. In wild-type cells this activity is probably counterbalanced by transglycosulases/transpeptidases involved in peptidoglycan synthesis (39). The membrane protein PotF2 was found to be down-regulated following initial adhesion, using the proteome approach. Interestingly, *potB* was up-regulated, suggesting differential regulation of membrane transporters during the initial stages of adhesion. PotB, part of the polyamine ABC transport system, was previously shown to be important for *A. tumefaciens* attachment to carrot suspension cells, and the resulting mutants were avirulent (42). Consistent with this report is the finding that the same gene, *potB*, was found to be surface induced in *P. putida*.

The subtractive hybridization approach indicated a possible change in bacterial organelles involved in motility, following initial adhesion to the surface. Type IV pili are used by bacterial pathogens to attach to epithelial cells and for twitching motility. Four genes involved in type IV pilus biogenesis, regulation, control, and secretion were found to be up-regulated within 6 h of attachment. These include a type IV fimbrial biosynthesis gene, *pilC*; the genes encoding the two-component response regulator PilR, a methyltransferase, PilK, and a component of the general secretion pathway, XcpS. The *xcp* gene products are essential for the transport and assembly of type IV pili by *P. aeruginosa* (5, 63). Immunoblot analysis was used to verify increased expression of one of these genes, *pilA*, following adhesion. Interestingly, the *pilK* gene is organized in a cluster encoding proteins which display homology to the enteric chemotaxis system Che. This Che-like network is thought to be involved in the control of twitching motility in response to environmental stimuli (13, 43). A role for type IV pili in surface sensing and biofilm structure was suggested by O'Toole and Kolter (49), since *P. aeruginosa* pili mutants impaired in twitching motility and microcolony formation were still able to attach to surfaces and form monolayers. Type IV pili have also been linked to the formation of cell clusters in *Myxococcus xanthus* (73, 74).

In contrast to the *pil* genes, genes involved in flagellum production were found to be down-regulated following initial

adhesion. These results were verified by immunoblot analysis for FliC. The primary function of flagella seems to be in initial cell-to-surface interactions since deletion of flagella or hyperflagellation leads to a dramatic reduction of attachment and bacterial surface coverage (41, 49). Nevertheless, after initiation of cell-surface contact, flagella seem to be dispensable, since immunoblot analysis revealed the absence of flagella in early biofilms. Furthermore, surface-induced repression of two flagellar genes with homology to the flagellar synthesis regulator *fleN* and the flagellar basal body rod protein *flgG* were found to be down-regulated within 6 h following attachment. The basal body, a multiprotein assembly that consists of four rings and an axial rod, is part of the rotary motor of the bacterial flagellum. Also found to be down-regulated upon attachment was the *nrqB* gene encoding the Na^{+}-translocating NADH:ubiquinone oxidoreductase. The sodium motive force required to power the rotary motor of the bacterial flagellum (which also drives other metabolic processes) is generated by the *Nrq* gene product, a unique, redox-driven sodium pump which functions as an entry point for electrons into the respiratory chain (37, 75). The identification of a negative control mechanism of flagellar synthesis is consistent with reports from a mucoid *P. aeruginosa* strain isolated from the cystic fibrosis-afflicted lung (29). In the mucoid strain the expression of the alternative sigma factor AlgT (AlgU) required for the synthesis of the exopolysaccharide alginate is linked to the down-regulation of the flagellar biosynthetic gene *fliC*. Surface-induced repression of flagellar synthesis has also been described for *Vibrio parahaemolyticus*. In this bacterium polar flagellar synthesis is repressed upon contact with a surface (7, 44). It is also interesting that, in *Yersinia pseudotuberculosis*, cell aggregation (clumping) in liquid medium is correlated to the absence of the major structural flagellin proteins FleA and FleB and a lack of motility (2). Besides the function of flagella in motility and initiation of cell-to-surface interactions, a second function of flagella in the developmental cycle of biofilm formation may be in detachment from the biofilm since flagella were detectable again in mature *P. putida* biofilms (3 to 7 days) by immunoblot analysis.

Molecular structures often associated with surface-attached bacteria involve the increased synthesis of extracytoplasmic polymeric substances. Alginate has been implicated as the embedding matrix in biofilms of *P. aeruginosa*. Three reports have shown that adherence of pseudomonads to a solid surface up-regulates the expression of the alginate biosynthetic genes *algC* (15, 16) and *algD* (35). The regulation of alginate is mediated by a hierarchy of proteins, including those encoded by the *algTmucABCD* operon. The *mucC* gene encodes a negative regulator for alginate biosynthesis (8, 46), and its expression was found here to be surface induced in *P. putida*.

It has been shown that the presence and composition of lipopolysaccharides that affect electrostatic interactions between bacteria and substratum contribute to the adhesiveness of *Pseudomonas* species (71). Thus, mutations in the lipopolysaccharide core (lipid A) biosynthesis genes of *E. coli* and *P. fluorescens* caused comparable reduction of bacterial adhesion (19, 31, 56). The requirement for increased adhesiveness is reflected in the surface-induced gene expression of two lipopolysaccharide biosynthesis genes, *lpxD* and *wbpG*, with *lpxD* encoding an enzyme that functions in lipid A biosynthesis and *wbpG* being essential for B-band lipopolysaccharide biosynthesis.

Increased antibiotic resistance is often associated with surface-attached bacteria and is attributed to antibiotic-modifying enzymes as well as to multidrug efflux pumps. The antibiotic resistance and virulence factor genes found here to be differentially regulated following initial adhesion, which require additional verification, include a component of the antibiotic efflux system, *mexB*; a streptomycin *str* resistance gene; β-lactamase *ampC*; and chitinase *chiA*. The expression of all four gene products was described to be surface induced (4, 6, 25, 32). Furthermore, a report from Espinosa-Urgel and coworkers (25) showed that *P. putida* KT2410, which carries a transposon insertion in a potential multidrug efflux pump, is defective in attachment to corn. Such a potential multidrug efflux pump has been recently identified as a pathogenicity factor in *Magnaporthe grisea*, a fungus responsible for rice blast disease (64). The authors concluded that *M. grisea* requires the up-regulation of specific ABC multidrug efflux pumps for pathogenesis, most likely to protect itself against plant defense mechanisms.

The establishment of the biofilm mode of growth is believed to be partially dependent on cell-to-cell signaling. Relative synthesis rates of AHLs were measured in biological samples, such as batch cultures, cystic fibrosis sputum, and mature biofilms using a radiometric technique (60). This approach demonstrated that two quorum-sensing signals ($3OC_{12}$-HSL and C_4-HSL) are generated in mature biofilms and in the sputum of a cystic fibrosis patient colonized by *P. aeruginosa* isolates. In the initial attachment experiment presented here, we were unable to detect signaling molecules in effluent supernatants within the time course of the experiment. On the protein level, only one protein, PotF2, could be correlated to quorum sensing. This putrescine ABC transport system has not been reported to be regulated by the *las* quorum-sensing system. Instead, the presence of $3OC_{12}$-HSL altered the expression of 15 different proteins that could not be assigned to adhesion, including nine proteins that had increased concentration due to AHL addition and six proteins that had decreased concentration. To our knowledge this is the first report of a negative regulated expression by the $3OC_{12}$-HSL signaling molecule (for a review, see reference 70). The experimental findings suggest that, in the case of early biofilm development, quorum sensing does not regulate the changes in the protein patterns and gene expression pattern and therefore is not responsible for the observed change in phenotype in *P. putida*. Thus, the changes must be a result of phenomena other than quorum-sensing signaling. This is consistent with reports showing that activation of *lasB*, a gene that is under the control of the LasR and LasI quorum-sensing system, occurs later in biofilm formation (22 h following initial attachment of *P. aeruginosa* [M. Parsek, personal communication]). Furthermore, Davies and coworkers (14) reported that cell-to-cell signaling is involved in *P. aeruginosa* biofilm maturation rather than initiation. This study showed that differentiation from planktonic bacteria into a fully mature biofilm was impaired in a LasI mutant, while initial stages of biofilm formation proceeded normally. This evidence links cell-to-cell signaling and biofilm maturation.

The results presented here give an overview of the important functions for surface colonization by *P. putida*. Although a

more detailed analysis of the identified genes and their specific role will be required, some conclusions can be drawn from this study. Cells attached to a surface undergo metabolic changes, since alterations in metabolic proteins and structural components such as membrane proteins and transporters occurred after initial adhesion to a surface. Some of these genes and gene products have not been previously described, thus indicating that we may have identified novel sets of genes necessary for attachment and novel elements of the physiology of *P. putida* when attached to a surface. One novel element might be the surface-related lag phase in their growth. Second, attachment to a surface induced a surface-regulated switch from flagellum-based motility to swarming or twitching motility. Two functions could be assigned to flagella in the developmental cycle of biofilm formation: (i) initiation of cell-surface contact, since following surface contact, flagella seem to be dispensable and (ii) detachment from the biofilm, since flagella were detectable again in mature *P. putida* biofilms. Third, the isolation of genes identified here with similarities to virulence factors, antibiotic resistance, and genes involved in bacterial adhesion to biotic surfaces such as carrot and corn suggests that initial colonization of abiotic and biotic surfaces such as host tissue proceeds via similar pathways. Fourth, in the case of early biofilm development, changes in protein patterns and gene expression patterns must be a result of signaling other than cell signaling by AHLs.

ACKNOWLEDGMENTS

We thank U. Völker for helpful discussions related to 2-D electrophoresis, C. Fuqua for providing the *A. tumefaciens* A136 and KYC6 strains, G. A. O'Toole for providing the *P. aeruginosa sad-110* PilA mutant, W. Shi and D. Wozniak for providing antibodies, and John Neuman for technical support.

This work was supported through cooperative agreement EEC-897039 between the National Science Foundation and Montana State University and by the industrial partners of the Center for Biofilm Engineering as well as by the Deutsche Forschungsgemeinschaft.

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