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Abiotic Surface Sensing and Biofilm-Dependent Regulation of Gene Expression in *Escherichia coli*

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To get further information on bacterial surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli* K-12, random insertion mutagenesis with Mu dX, a mini-Mu carrying the promoterless *lacZ* gene, was performed with an *ompR234* adherent strain, and a simple screen was developed to assess changes in gene expression in biofilm cells versus planktonic cells. This screen revealed that major changes in the pattern of gene expression occur during biofilm development: the transcription of 38% of the genes was affected within biofilms. Different cell functions were more expressed in sessile bacteria: the OmpC porin, the high-affinity transport system of glycine betaine (encoded by the *proU* operon), the colanic acid exopolysaccharide (*wca* locus, formerly called *cps*), tripeptidase T (*pepT*), and the nickel high-affinity transport system (*nikA*). On the other hand, the syntheses of flagellin (*fliC*) and of a putative protein of 92 amino acids (*f92*) were both reduced in biofilms. Such a genetic reprogramming of gene expression in biofilms seems to result from changes in multiple environmental physicochemical conditions. In this work, we show that bacteria within biofilms encounter higher-osmolarity conditions, greater oxygen limitation, and higher cell density than in the liquid phase.

Many bacteria can attach to solid surfaces and form biofilms, which are defined as matrix-enclosed microbial populations adherent to each other and to surfaces or interfaces (11). Biofilm formation is such a common phenomenon that virtually every material that comes into contact with naturally occurring fluids, such as blood or seawater, becomes rapidly covered with bacteria (8, 10). Given the important medical and economic consequences of this detrimental situation, understanding the colonization process would help in the design of surface coating methods able to prevent biofilm formation.

Abundant evidence indicates that cells grown on solid surfaces within biofilms are in a physiological state different from that of planktonic cells (reviewed in reference 22). The most difficult properties of biofilm bacteria are their extreme resistance to treatment with biocides and detergents and their high tolerance to prolonged antibiotic therapy in human infections (19, 26). Bacterial structures involved in biofilm formation have been well characterized for some bacteria; AtlE (an autolysin) in *Staphylococcus epidermidis* (25), type I pili (42) and curli (56) in *Escherichia coli*, and type IV pili in *Pseudomonas aeruginosa* (41) have been described as major structures for interaction with the surface. The importance of flagellar motility in initiation of biofilm development in *Pseudomonas fluorescens*, *P. aeruginosa*, and *E. coli* was also reported (40–42).

However, there is still limited information available on bacterial surface sensing at the gene expression level. Surface-induced expression of the *laf* genes, which are responsible for the synthesis of peritrichous lateral flagella, was correlated with a decreased rotation ability of the unique polar flagellum in *Vibrio parahaemolyticus* (6, 35) and *Proteus mirabilis* (5). Specific induced expressions of the *P. aeruginosa* genes *algC* (13, 14) and *algD* (27) and of the *sfaA* gene (encoding S fimbrial adhesins) of a pathogenic strain of *E. coli* (47) were also observed after contact of the bacteria with a solid surface, but the surface-sensing mechanisms were not investigated. Furthermore, in many bacterial species, regulation of metabolic functions, particularly those relating to virulence, involves cell-to-cell signalling molecules (such as *N*-acylhomoserine lactones in gram-negative bacteria). These signal molecules accumulate in the bacterial environment as a function of cell number. Thus, the high density of bacteria within biofilms led to the hypothesis that cell-to-cell signal mechanisms may play an important role in the establishment of the biofilm-specific physiological state. Evidence in support of this hypothesis has been recently provided. Acylhomoserine lactones were detected in biofilms formed on urethral catheters removed from patients (52) and on immersed stones from the San Marcos river in Texas (36). Moreover, Davies and coworkers (15) demonstrated that the LasI-LasR system (one of the two cell-to-cell signalling systems of *P. aeruginosa*) is required for the normal development of biofilms.

The present work was undertaken in the very well characterized *E. coli* K-12 context in order to identify biofilm-regulated functions and to gather information on the regulation processes triggered by bacterial contact with the surface. Using a new, reliable system to assess changes in gene expression in biofilm cells versus planktonic cells, we found that 38% of the *E. coli* genes are differentially expressed in the biofilm. In addition to cell-to-cell signalling mechanisms, microenvironmental conditions of osmolarity and oxygen concentration can be correlated with this major change in gene expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All of the *E. coli* K-12 strains and plasmids used in this work are listed in Table 1. The bacteria were grown in complete Luria-Bertani (LB) medium (37) or in minimal M63 medium (37) supplemented with mannitol (0.2%) or glucose (0.2%) as carbon sources. MOPS (morpholinepropanesulfonic acid) medium supplemented with glycerol (0.4%) was prepared as described previously (39). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; tetracycline, 10 μg/ml; and chloramphenicol, 20 μg/ml. For all experiments with derivatives of strain YK3421, histidine (50 μg/ml), thymine (25 μg/ml), uracil (100 μg/ml), and cytosine (25 μg/ml) were added to the M63 medium.

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TABLE 1. *E. coli* K-12 strains and plasmids used

Strain or plasmid	Description	Source or reference
Strains		
CSH135	F′ *lacZ*::Mu *cts*62 Mu dX Δ(*gpt-lac*) *his met tyr gyrA rpsL*	38
DH5α	Δ*lacU169 recA1 endA1 gyrA96 relA1 hsdR17 thi-1 supE44*	Laboratory collection
GM37	MC4100 Φ(*proU-lacZ*)*2*(Hyb) (λp *lac* Mu15)	34
HYD723	MC4100 *nikA* (*hydC*)::Mu dI(Ap^r *lac*)	57
MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Laboratory collection
MG1655	λ$^-$ F$^-$ prototroph	Laboratory collection
MH225	MC4100 Φ(*ompC-lacZ*$^+$)*10-25*	21
PHL628	MG1655 *malA-kan ompR234*	56
PHL644	MC4100 *malA-kan ompR234*	56
PHL665	PHL644 *pepT*::*lacZ* (Mu dX)	This study
PHL698	YK3421 *malA-kan*	This study
PHL699	YK3421 *malA-kan ompR234*	This study
PHL701	SG20781 *malA-kan ompR234*	This study
PHL702	SG20781 *malA-kan*	This study
PHL744	MC4100 *malT54*::Tn*10 ompR234*	56
PHL789	MC4100 *pepT*::*lacZ* (Mu dX) *malT54*::Tn*10*	This study
PHL801	MC4100 *pepT*::*lacZ* (Mu dX) *ompR331*::Tn*10*	This study
PHL825	PHL644 *f92*::*lacZ* (Mu dX)	This study
PHL826	SG20781 *ompR331*::Tn*10*	This study
PHL840	MH225 *malA-kan ompR234*	56
PHL861	MC4100 *f92*::*lacZ* (Mu dX) *ompR331*::Tn*10*	This study
PHL863	GM37 *malT54*::Tn*10 ompR234*	This study
PHL864	GM37 *malT54*::Tn*10*	This study
PHL865	GM37 *ompR331*::Tn*10*	This study
PHL868	MC4100 *f92*::*lacZ* (Mu dX) *malT54*::Tn*10*	This study
PHL923	YK3421 *ompR331*::Tn*10*	This study
PHL965	HYD723 *malA-kan ompR234*	This study
PHL1036	SG20781 pCP994 (Ap^r)	This study
PHL1064	GM37 pCP994 (Ap^r)	This study
SG20781	MC4100 *wcaB10* (*cpsB10*)::*lac*-Mu-*imm*λ	7
TK821	MC4100 *ompR331*::Tn*10*	21
YK410	F$^-$ *araD139* Δ*lacU169 rpsL thi nalA thyA pyrC46 his*	31
YK3421	YK410 *fliC* (*hag*)::Mu d(Ap^r *lac*)	31
Plasmids		
pCP752	pPH126 (51) with a 14-kb *Pst*I fragment cloned in *Nsi*I and harboring a genomic fragment (5 kb) with the *pepT* gene fused to the *lacZ* gene from Mu dX	This study
pCP832	pBR322 with a 16-kb *Pst*I fragment harboring a genomic fragment (7 kb) with the *f92* gene fused to the *lacZ* gene from Mu dX	This study
pCP994	pKK233-2 (2) with a 697-bp fragment containing the *csgD* ORF	This study

Genetic methods. Random insertion mutagenesis with phage Mu dX (4) carrying the promoterless *lacZ* gene was performed by the procedure described by Miller (38). Phage P1 *vir* was used for transductions, which were carried out as described by Miller (37). The *ompR234* mutation was transferred by using its genetic linkage (cotransduction at 50%) with *malA* or *malT* followed by screening of adherent transductants in 24-well microtitration plates.

Screening of *lacZ* fusions with altered expression within biofilms. Mutant clones obtained by transposition of Mu dX were grown in 200 μl of M63-mannitol (0.2%) medium in the wells of a 96-well microtitration plate. After 24 h of incubation at 30°C, a visible biofilm was present on the wall of each well. The medium containing the free-living bacteria of each well was removed carefully and was introduced into the corresponding well of a precooled microtitration plate, whereas attached bacteria were suspended in 200 μl of cold M63-mannitol medium by vigorous shaking of the plate. The turbidity (optical density at 630 nm [OD_{630}]) and the initial absorbance of suspensions at 405 nm were estimated by using a microplate photometer. Bacteria were then permeabilized by the addition of 20 μl of a permeabilization reagent (48), and *o*-nitrophenyl-β-D-galactoside was added to a final concentration of 2.7 mM. Quantification of β-galactosidase activity was performed at 405 nm with a microplate photometer after various periods of incubation at room temperature. The ratio between the OD_{405} and OD_{630} was calculated for the free-living and biofilm bacteria (44).

Monitoring of *lacZ* fusion expression within biofilms developed on inert surfaces. The wells of 24-well microtitration plates, petri dishes, or glass tubes containing Plexiglas strips were filled with minimal M63 medium and inoculated with about 5×10^6 cells of an overnight culture of the clone of interest. After different incubation times at 30°C, the liquid medium containing free-living bacteria was carefully removed; biofilms developed on inert surfaces were washed, and sessile bacteria were suspended in cold medium by pipetting up and down. Cell biomass was estimated by OD_{600} measurements, and β-galactosidase assays were performed as described below.

To be sure that no alteration of absorbance values occurred due to cell clumps remaining after resuspension and dispersal, both sessile and planktonic cell suspensions were stained with a 2-μg/ml solution of 4′,6-diamidino-2-phenylindole (DAPI) and visualized by epifluorescence microscopy. No more cell clumps were observed in either situation. Furthermore, the total amounts of protein per OD_{600} unit were estimated, at different times, for both sessile and planktonic populations of strain PHL644 grown in M63-mannitol medium in petri dishes. Protein amounts were determined after cell lysis in the presence of 1% sodium dodecyl sulfate by using the bicinchoninic acid protein assay reagent, as recommended by the manufacturer (Pierce), with gamma globulin as a standard. Equal ratios were obtained for free-living and attached bacteria (10 independent measurements). One OD_{600} unit (1-cm path length, 1 ml) contains 0.16 mg of total proteins for both populations.

DNA manipulations. Standard techniques were used for chromosomal DNA preparation, plasmid extraction, gel electrophoresis, and DNA sequencing (45). Restriction endonucleases and T4 DNA ligase were used as recommended by the manufacturers.

Construction of plasmid pCP994. A 697-bp fragment containing the *csgD* open reading frame (ORF) was amplified by PCR with chromosomal DNA of strain MC4100 as the template and two primers (5′-CCGCCACACTGCAGCGTAA ATAACG-3′, and 5′-CGGGGTTTCATCATGACTAATGAAG-3′) overlapping the *csgD* ORF and containing *Pst*I and *Bsp*HI cutting sites, respectively. The PCR fragment was digested with *Pst*I and *Bsp*HI and then purified and cloned in the *Pst*I and *Nco*I sites of the plasmid cloning vector pKK233-2 (Ap^r) (2).

Identification of genes affected by Mu dX insertion. The chromosomal DNAs of mutants were extracted and digested with *Pst*I, and fragments of more than 10

kb were cloned in pBR322 or pPH126 (51). Transformants displaying β-galactosidase activity were analyzed. The chromosomal region adjacent to the *lacZ* end of the Mu genome was sequenced with a 20-bp *lacZ*-targeted oligonucleotide (5′-CAGGCATCAGGATTTGTGGC-3′).

β-Galactosidase assay. β-Galactosidase specific activity in toluenized samples was measured by spectrophotometrically monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactoside into *o*-nitrophenol at 420 nm and 37°C (37) and was expressed as units per milligram of protein, where 1 U corresponds to 1 nmol of product liberated per min.

Potassium assay. Cells from 1-ml portions of suspensions of free-living and sessile bacteria grown in M63-mannitol medium in petri dishes, and containing the same number of cells (10^8), were harvested by centrifugation for 30 s, the supernatants were removed, and the pellets were suspended in 1 ml of deionized water. The suspensions were boiled for 1 min to release potassium from the cells, and the potassium concentrations were determined by flame photometry (55).

Preparation of conditioned medium. An actively growing culture of *E. coli* DH5α was diluted 1,000-fold in LB medium and grown for 24 h at 37°C to a final OD_{600} of 3.5. Cells were removed by centrifugation for 15 min at 4°C. The supernatant was sterilized by filtration through a 0.2-μm-pore-size filter and stored at −80°C. When conditioned medium was used to support growth, tryptone and yeast extract were added to concentrations of 10 and 5 g/liter, respectively (20). Fifty milliliters of conditioned medium or fresh LB medium was inoculated at a final dilution of 1/100 with a stationary-phase culture of the appropriate strain. The cultures were incubated at 30°C with vigorous shaking. The conditioned medium supported growth to a cell division rate equal to that of cells grown in fresh LB medium.

Electron microscopy. Cell cultures were grown in M63-mannitol medium at 30°C in gently shaken glass tubes, each containing one plastic strip (attached bacteria) or not containing a strip (free-living bacteria). At different incubation times between 8 and 48 h, suspensions of free-living bacteria and attached bacteria were carefully recovered to prevent breakdown of flagella and were allowed to adhere to carbon-coated 200-mesh grids. After being stained with 1% phosphotungstic acid (Sigma), the grids were examined with a Philips CM120 electron microscope.

RESULTS

A major change in the pattern of gene expression occurs in *E. coli* biofilms. Random insertion mutagenesis with Mu dX, a bacteriophage Mu derivative carrying the promoterless *lacZ* reporter gene (4), was performed on the biofilm-forming *E. coli* K-12 strain PHL644, and, for 885 clones, the β-galactosidase specific activities of the biofilm population and the nonattached cells were compared in microtitration plates (see Materials and Methods). A total of 446 clones were Lac^+, and three classes of *lacZ* fusions could be distinguished. Ninety-eight fusions belong to the first class, corresponding to fusions significantly (1.5- to 10-fold) more expressed in the biofilm cells (up-regulated fusions). In the second class, 73 fusions were significantly (2- to 10-fold) less expressed in the biofilm (down-regulated fusions). Most of the fusions (275) belong to the third class, corresponding to invariant fusions. A total of 38% of the fusions, therefore, were differently expressed in biofilm. A second β-galactosidase assay was conducted on a representative sample of 36 clones to compare the levels of expression in biofilm and free-living cells grown in 24-well microtitration plates (see Materials and Methods). The results supported those of the first analysis.

The higher osmolarity encountered in biofilm triggers major changes in gene expression. As detailed by Goodman and Marshall (22), a bacterium approaching a solid-water interface could encounter a gradient of inorganic ions and organic ionized molecules (attracted to counterbalance the electric charges existing at the solid surface), alterations in surface free energy, a lower pH level (resulting from proton accumulation), modified viscosity and osmolarity, and altered rates of gas exchange. Among these conditions, we chose to focus first on osmolarity.

It is well established that in *E. coli*, the intracellular content of potassium ions varies proportionally to the external osmotic pressure (12, 18). Intracellular K^+ concentrations were measured for attached and planktonic PHL644 bacteria in 20 ml of liquid M63-mannitol medium introduced in petri dishes and incubated at 30°C without agitation (see Materials and Methods). After 10 h of incubation at 30°C, the intracellular potassium content in free-living bacteria was 1.81 ± 0.08 mmol/g of protein, and that in attached bacteria was 2.84 ± 0.28 mmol/g of protein (means ± standard errors for 10 repetitions; $P < 0.001$ by Student's *t* test). This corresponds to 223 ± 10 and 349 ± 34 mmol/liter for free-living and attached bacteria, respectively, where the cell volume was estimated to 1.2 μm^3 for both by electron microscopy observations (more than 10 independent measurements; this result is in good agreement with data reported previously [17]). These results suggest that attached bacteria indeed encounter higher-osmolarity conditions than planktonic cells from the beginning of the colonization process.

To study the effects of osmolarity further, the expression of four genes known to be osmoregulated was compared for attached and free-living bacteria grown in petri dishes, as described in Materials and Methods. The porin *ompC* gene (46); the *proU* operon, which encodes a high-affinity glycine betaine transport system (23); and the *wcaB* gene (formerly called *cpsB*), which is involved in the synthesis of the capsular exopolysaccharide colanic acid (50) are up-regulated by high osmolarity. On the other hand, the flagellin gene *fliC* is down-regulated by high salt concentrations (49). For each fusion up-regulated by osmolarity, a clear induction (two- to threefold) was observed in sessile populations during the first 40 h (Fig. 1A, B, and D). The down-regulated fusion *fliC* is consistently less expressed in biofilm bacteria (Fig. 1C). Similar weak differences in *fliC-lacZ* expression in the presence of regulatory mutations conferring a flagellum-deficient phenotype have been reported (30). This suggests that flagellum synthesis is reduced in biofilms, even if the level of *fliC* expression is far from being totally abolished. An electron microscopic study (see Materials and Methods) revealed that biofilm cells of the motile strain PHL628 did not synthesize flagella (and produced a copious amount of curli); no flagella were visualized on sessile bacteria and no broken flagella were released in biofilm cell suspensions (Fig. 2B), whereas flagellated free-living bacteria were easy to visualize (Fig. 2A). Synthesis of curli was lower in these last cells (43).

To identify new genes regulated both by biofilm state and osmolarity, 40 clones isolated by the insertion mutagenesis reported above and showing biofilm regulation of their *lacZ* fusions were tested for osmoregulation. This screening was performed on M63-glucose agar plates, supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), in the presence or absence of NaCl (0.3 M). Differences in the intensity of the blue coloration permitted the isolation of PHL825, a clone containing a biofilm-down-regulated fusion which is repressed by a high salt concentration. A kinetic analysis of the expression of this fusion confirmed its biofilm regulation (Fig. 3A). To determine the precise extent of osmoregulation, the expression of the fusion in shaken liquid cultures containing different salt concentrations was monitored (Fig. 3B); a fourfold-lower expression was observed at high osmolarity (in the presence of 0.3 M NaCl). To identify the target gene of the Mu dX insertion, the DNA fragment surrounding the *lacZ* gene was cloned on a multicopy plasmid to give pCP832 and sequenced (see Materials and Methods). A BLAST search identified this gene as the uncharacterized *f92* ORF (ECAE000245; min 33.5), encoding a putative short protein (92 amino acids).

Effects of the *ompR234* allele. Since wild-type *E. coli* K-12 strains are not able to attach to surfaces, the experiments reported above were done in the *ompR234* genetic background. EnvZ-OmpR is a two-component signal transduction

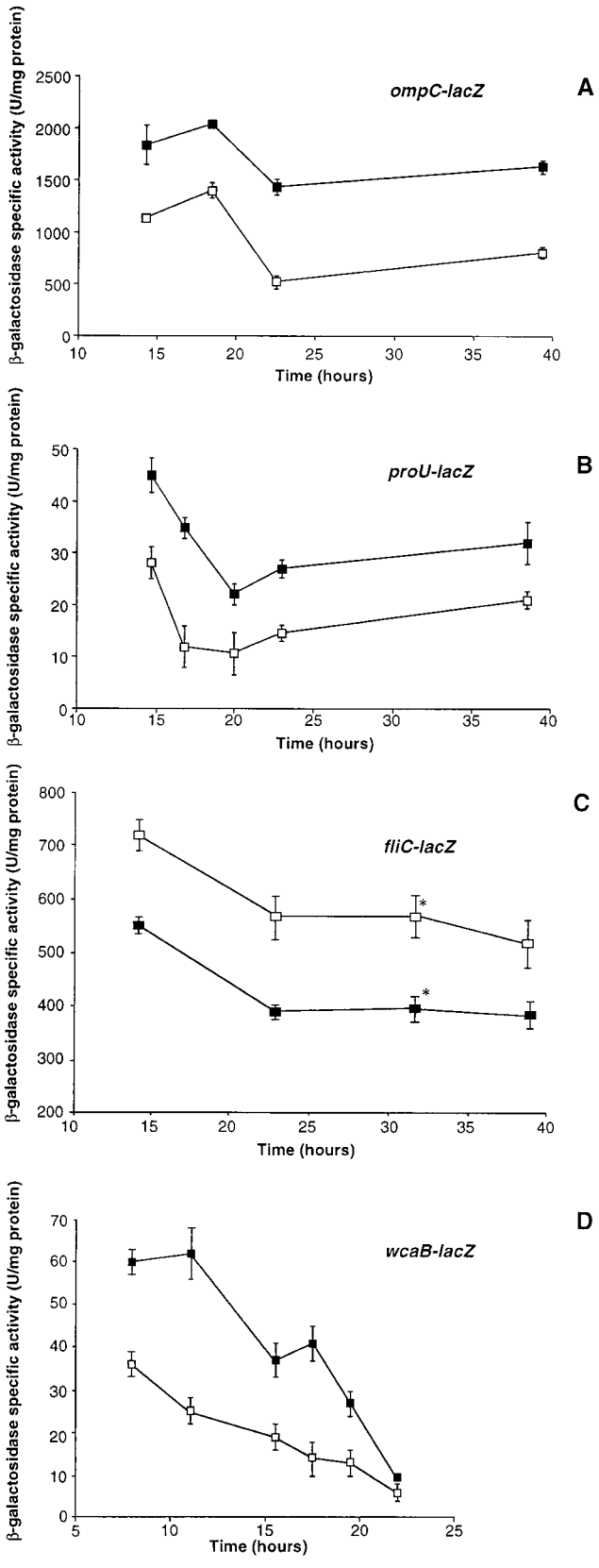

FIG. 1. Kinetics of osmoregulated *lacZ* fusion expression in attached and free-living bacteria. Specific β-galactosidase activities of *ompC-lacZ* (A), *proU-lacZ* (B), *fliC-lacZ* (C), and *wcaB-lacZ* (D) fusions were measured in free-living (open squares) and sessile (filled squares) bacteria during biofilm development on petri dishes in M63-mannitol medium (A, B, and C) or on Plexiglas strips in M63-glucose medium (D) as described in Materials and Methods. Results are means ± standard deviations of 3 or 10 (*) independent measurements.

system involved in bacterial osmoregulation. The *ompR234* allele increases the expression of *csgA*, the curlin-encoding gene (the resulting overproduction of curli confers the adherence properties), and of porin genes *ompC* and *ompF* (56). Although the osmoregulation mediated by EnvZ-OmpR is still efficient in *ompR234* strains (56), the effect of this allele on the biofilm-regulated fusions had to be examined. The expressions of *f92-lacZ*, *fliC-lacZ*, *wcaB-lacZ*, and *proU-lacZ* in wild-type, *ompR234*, and *ompR*::Tn*10* strains in planktonically grown cultures were compared (Table 2). For all of these strains, similar growth rates were observed (data not shown). The expression of *proU*, a gene which is osmoregulated independently of the EnvZ-OmpR system (32), was similar in the three backgrounds. The same results were observed for *fliC*, whereas *f92* and the colanic acid gene *wcaB* were strongly dependent on the *ompR* allele. This last result indicates that these two genes belong to the OmpR regulon.

To control for the possibility that the differences in gene expression observed for the attached and the planktonic bacteria do not result from an unexpected effect of the *ompR234* allele, the differential expression of the two osmoregulated fusions *proU-lacZ* (EnvZ-OmpR independent) and *wcaB-lacZ* (EnvZ-OmpR dependent) was assessed in an *ompR*$^+$ background. For these experiments, bacterial adherence properties were obtained by overexpression of the *csgD* gene (a specific activator of the *csgBA* operon [24]) cloned on a multicopy plasmid to give pCP994 (see Materials and Methods). *ompR*$^+$ strains harboring this plasmid adhered to surfaces with the same effectiveness as *ompR234* strains and formed biofilms as thick as those formed by *ompR234* strains (20 μm after 24 h). As shown in Fig. 4, the same differences in expression as in the *ompR234* background were observed. These results validate the approach used for identifying loci whose expression changes in planktonic versus biofilm-grown cells.

Cells in biofilm also encounter limited diffusion of oxygen. Because of the particular physicochemical conditions on uncolonized surfaces and the high cellular density in established biofilms, oxygen diffusion could be an important parameter to explain differential gene expression in attached and free-living bacteria. Furthermore, in all of the experiments reported above, the microtitration and petri dishes were incubated in the absence of agitation. Due to the small total amount present in the liquid medium, the local oxygen availability could rapidly become a discriminating factor between the bacteria colonizing the bottom of the vessel and the cells swimming in the liquid phase. A nitrate assay (28) performed on strain PHL644 cultivated in liquid medium in unshaken petri dishes indicated that microaerophilic conditions were reached after 16 h (5 mM nitrite was detected in the medium). *nikA* encodes a high-affinity nickel transport system, and the expression of this gene is known to be precisely tuned by the level of oxygen availability (57). The *nikA-lacZ* fusion of strain HYD723 was introduced in an *ompR234* background (to give strain PHL965), and its expressions in biofilm and planktonic bacteria under standard incubation conditions were compared. A fivefold-higher expression was observed in biofilm after 23 and 45 h (Fig. 5A). These differences were not observable in anaerobiosis (Fig. 5B), indicating that the local oxygen concentration is the main parameter responsible for the differential *nikA* expression under the standard conditions.

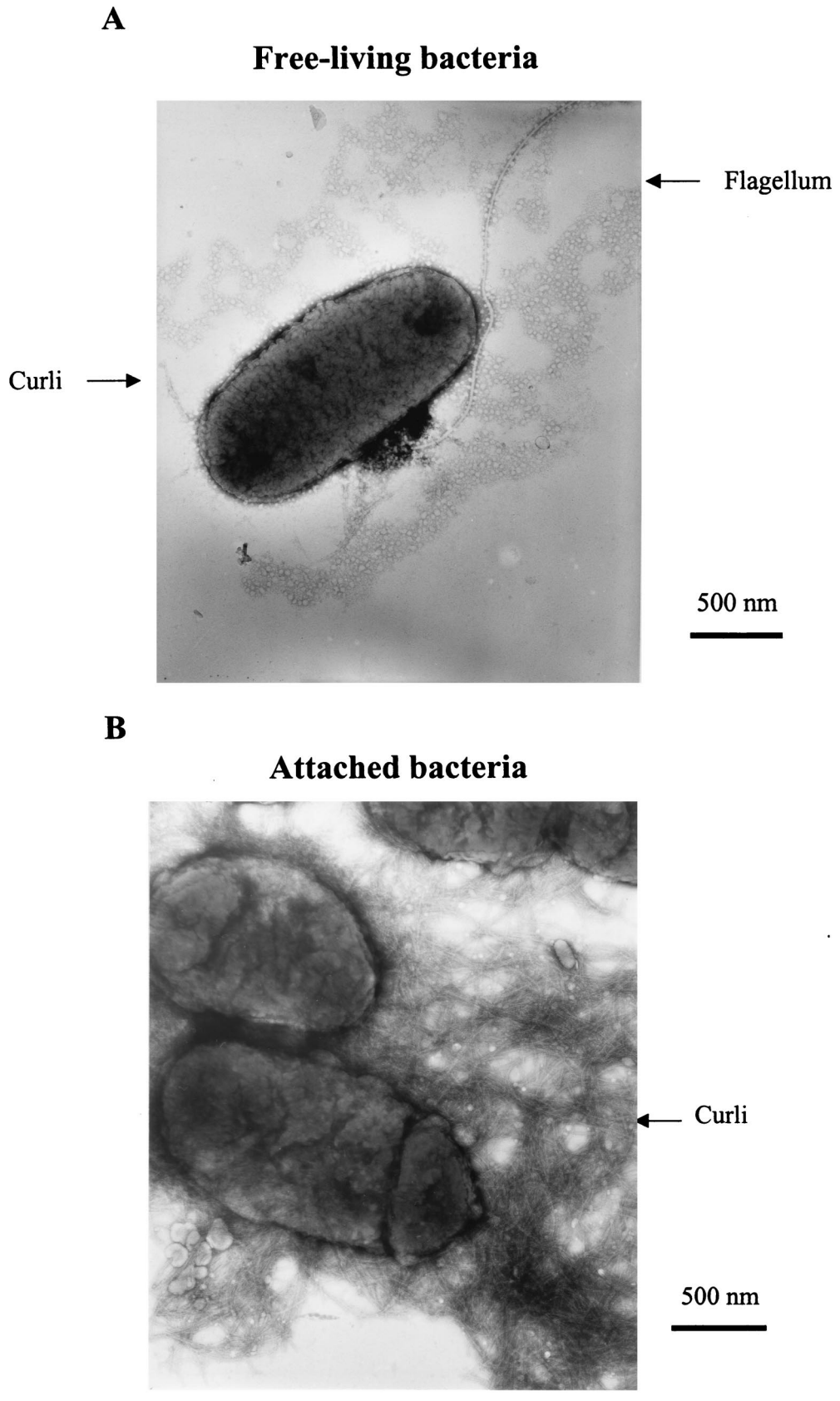

FIG. 2. Electron micrographs of negatively stained bacteria of planktonic (A) or biofilm (B) cell suspensions of the motile PHL628 strain. The cells were grown in M63-mannitol medium, and biofilms were formed on plastic strips. The micrographs, corresponding to observations made after 24 h of culture, are representative of several microscopic observations performed at between 8 and 48 h of biofilm development.

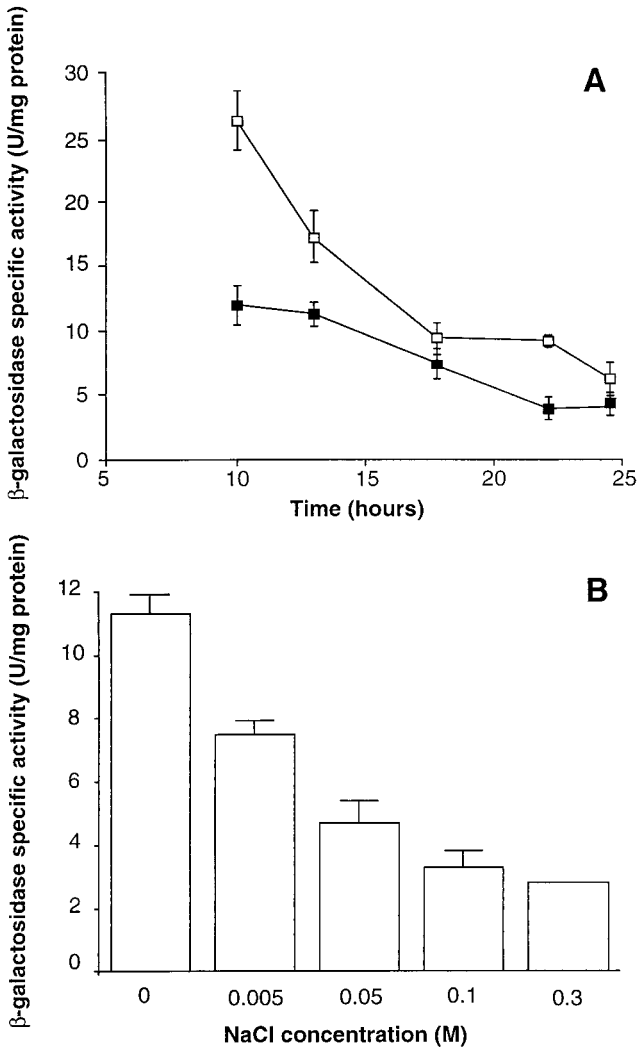

FIG. 3. Comparison of down-regulated *lacZ* fusion expression (strain PHL825) in attached and free-living bacteria during biofilm development on petri dishes (A) and in shaken liquid cultures under different osmolarity conditions (B). (A) Biofilm cultures were performed on petri dishes in M63-mannitol medium, and specific β-galactosidase activities in free-living (open squares) and sessile (filled squares) bacteria were measured as described in Materials and Methods. Results are means ± standard deviations of three independent measurements. (B) Cells were grown at 30°C in low-osmolarity MOPS-glycerol medium supplemented with increasing NaCl concentrations of 0 to 0.3 M. Two independent measurements of specific β-galactosidase activities were performed on mid-exponential-growth-phase cells. Results are means ± standard deviations of two independent measurements.

To identify other genes regulated by both biofilm and anaerobiosis, the expressions of a sample of 48 *lacZ* fusions on solid LB medium supplemented with X-Gal during aerobiosis and anaerobiosis were compared. A clone (PHL665) carrying a biofilm-up-regulated fusion that was highly expressed in anaerobiosis was isolated. After cloning and sequencing of the chromosomal region in contact with the end of Mu dX (plasmid pCP752), a BLAST search identified the target gene as *pepT* (min 25.5). This gene encodes aminotripeptidase T, which is able to remove the N-terminal amino acid from various tripeptides (53). The expression of the *pepT-lacZ* fusion was monitored during biofilm development in petri dishes during anaerobiosis and under standard conditions (Fig. 6). A consistently higher level of expression was observed in attached bacteria from 12 to 33 h of incubation under standard conditions (Fig. 6A). This result confirms the up-regulation of *pepT* in sessile bacteria. A comparison of *pepT* expression under anaerobic and standard conditions revealed a slightly but significantly higher expression in the two cell populations during anaerobiosis (Fig. 6B). This induction of the *E. coli pepT* gene in anaerobiosis is in good agreement with the positive regulation by oxygen limitation of the *pepT* locus reported for *Salmonella typhimurium* (53). However, in contrast to the *nikA-lacZ* fusion, *pepT-lacZ* remained differently expressed in attached and free-living bacteria in the absence of oxygen; similar results were obtained for the *f92*, *ompC*, and *proU* osmoregulated fusions (Table 3). Although microaerophilic conditions seem to be one of the factors implicated in regulation of gene expression in biofilms, differences in oxygen availability are therefore not responsible for the differential expression of these four genes.

Cell-to-cell signalling. Since we have shown that osmolarity is a key factor in biofilm-dependent gene regulation, a possible osmoregulation of *pepT* was examined. Similar levels of expression of the *pepT-lacZ* fusion were obtained in liquid culture in MOPS-glycerol medium (126 U/mg of protein) and in MOPS-glycerol containing 0.3 M NaCl (118 U/mg of protein), indicating that *pepT*, in contrast to *f92*, *ompC*, *fliC*, *wcaB*, and *proU*, is not osmoregulated. In addition, a similar expression was observed in the *ompR*$^+$, *ompR234*, and *ompR*::Tn*10* backgrounds (Table 2). Kinetic analysis of the expression of the *pepT-lacZ* fusion during growth in liquid M63-mannitol medium gave some clues concerning its regulation. A sudden increase in β-galactosidase activity was measured at the end of the exponential phase (Fig. 7A), probably resulting from a cell density or starvation signalling mechanism. As a cell-to-cell signalling mechanism has been shown to be involved in *P. aeruginosa* biofilm development (15), a quorum-sensing mechanism was investigated for *pepT* regulation. Conditioned medium (i.e., medium containing the suspected signalling factors) was removed from a stationary-phase culture of strain DH5α, as described previously (3, 20) (see Materials and Methods) and used to support growth of the *pepT-lacZ* strain. Overex-

TABLE 2. Comparison of biofilm-regulated *lacZ* fusions in wild-type *ompR*, *ompR234*, and *ompR* null contexts[a]

Strain	Relevant genotype	β-Galactosidase sp act (U/mg of protein)[b]
PHL789	*pepT-lacZ*	91
PHL665	*pepT-lacZ ompR234*	96
PHL801	*pepT-lacZ ompR*::Tn*10*	99
PHL868	*f92-lacZ*	20
PHL825	*f92-lacZ ompR234*	17
PHL861	*f92-lacZ ompR*::Tn*10*	46
PHL698	*fliC-lacZ*	1,537
PHL699	*fliC-lacZ ompR234*	1,309
PHL923	*fliC-lacZ ompR*::Tn*10*	1,375
PHL702	*wcaB-lacZ*	57
PHL701	*wcaB-lacZ ompR234*	12
PHL826	*wcaB-lacZ ompR*::Tn*10*	469
PHL864	*proU-lacZ*	57
PHL863	*proU-lacZ ompR234*	67
PHL865	*proU-lacZ ompR*::Tn*10*	71

[a] Bacteria were grown at 30°C in shaken M63 liquid medium supplemented with mannitol (or glucose for strains carrying the *wcaB-lacZ* fusion).

[b] β-Galactosidase activity was assayed at mid-log growth phase. The values are representative of those from three independent assays.

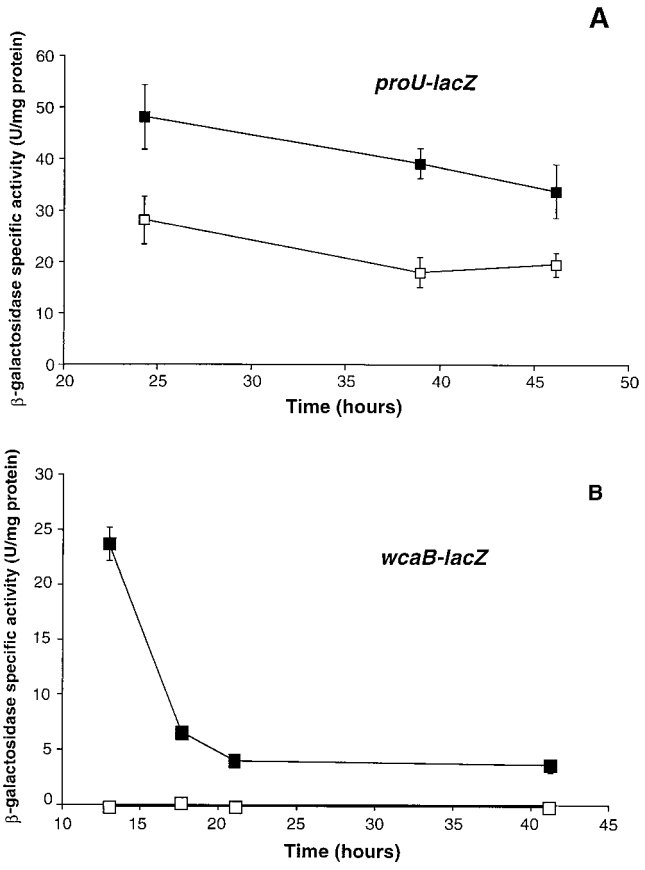

FIG. 4. Kinetics of *proU-lacZ* (A) and *wcaB-lacZ* (B) fusion expression in attached and free-living bacteria of adherent *ompR* wild-type strains (PHL1064 and PHL1036, respectively) overproducing the CsgD specific activator of the curlin-encoding *csgBA* operon. Specific β-galactosidase activities were measured in free-living (open squares) and sessile (filled squares) bacteria during biofilm development on petri dishes in M63 medium supplemented with ampicillin and mannitol (A) or glucose (B), as described in Materials and Methods. Results are means ± standard deviations of three independent measurements.

pression of the fusion was observed at a low cell density (Fig. 7B), confirming the emission of some cell-to-cell signalling factors in the conditioned medium in response to high cell density. As the cellular density is far higher in our biofilm (10^{11} cells/ml are reached in a 20-μm-thick biofilm after 24 h) than in the liquid phase (10^9 cells/ml), we can suggest that the biofilm-dependent activation of *pepT* resulted from a cell-to-cell signalling mechanism.

DISCUSSION

In natural environments, bacteria are often found as sessile communities known as biofilms (8, 10). To date, the bacterial structures of adherence (25, 40–42, 56) and the physiological processes involved in bacterial surface colonization (15, 40, 41) are better understood than the genetic responses of bacteria adhering to a surface. By using a library of *lacZ* fusions and a reliable screen for identifying genes whose expression changes in biofilm versus planktonic cells, the transcription of 38% of the *E. coli* genes was shown to be modified during the colonization process. Several genes with altered expression in biofilms were identified. Different cellular functions were induced in attached bacteria: the OmpC porin, the high-affinity transport system of glycine betaine, colanic acid production (the *E. coli* class I exopolysaccharide), tripeptidase T, and synthesis of a nickel high-affinity transport system. On the other hand, the syntheses of flagella and of a putative protein of 92 amino acids were both reduced in biofilms. The induction of colanic acid synthesis at the gene expression level is consistent with the results of other authors, who have described a similar surface activation of exopolysaccharidic alginate genes in *P. aeruginosa* (13, 14, 27). These results emphasize the major role of exopolysaccharides in biofilm development, as previously reported (1, 9). Another important aspect of the biofilm formation process is triggered by flagella in different organisms, such as *P. fluorescens*, *P. aeruginosa*, and some strains of *E. coli* (40–42). However, in biofilms of *E. coli* K-12 strains overproducing curli, no flagella are produced (Fig. 2B). Furthermore, nonmotile strains (such as adherent derivatives of MC4100 overproducing curli) form thick biofilms as well as motile strains (such as adherent derivatives of MG1655 overproducing curli) do. It seems that, depending on environmental conditions and biofilm communities, different approaches and cell surface structures such as flagella, pili, S-fimbrial adhesins, autolysins, and curli could be used to initiate biofilm formation (25, 41, 42, 47, 56).

The surprising major change in gene expression observed within *E. coli* biofilms is consistent with the results of several teams that have shown that new protein synthesis is required for biofilm formation (40) and that patterns of proteins syn-

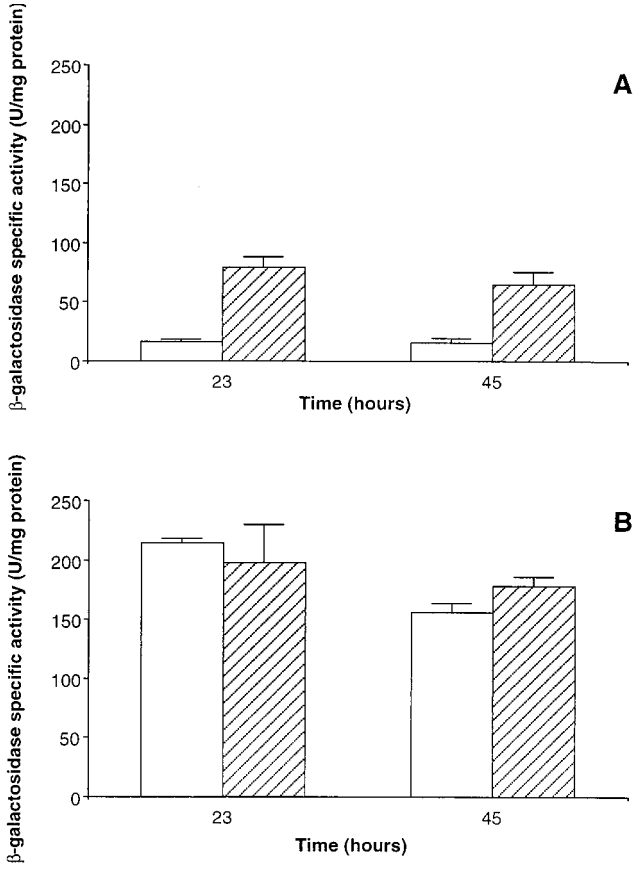

FIG. 5. Comparison of *nikA-lacZ* expression in attached and free-living bacteria grown under standard conditions (A) or in anaerobiosis (B). Biofilm cultures were performed in M63-mannitol medium on petri dishes incubated in Generbox anaerobic jars (B) or not (A) for 23 and 45 h and specific β-galactosidase activities were measured in free-living (open bars) and sessile (hatched bars) bacteria as described in Materials and Methods. Results are means ± standard deviations of three independent measurements.

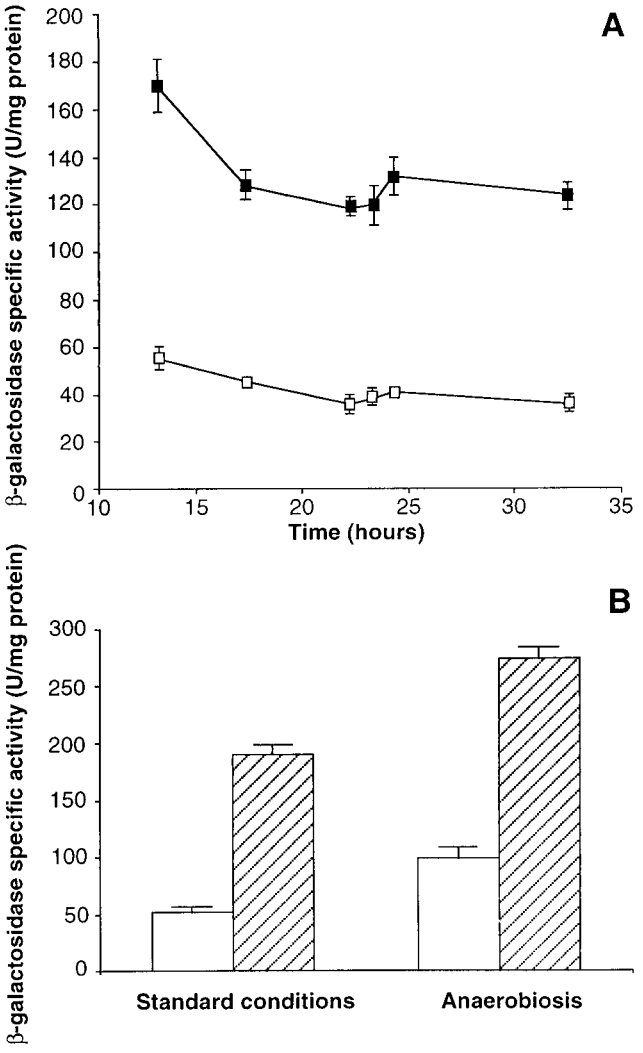

FIG. 6. Kinetics of up-regulated *lacZ* fusion expression (strain PHL665) in attached and free-living bacteria (A) and comparison of fusion expression under standard conditions and during anaerobiosis after 28 h of biofilm cultures (B). Biofilm cultures were performed in M63-mannitol medium on petri dishes, and specific β-galactosidase activities were measured in free-living bacteria (open squares and bars) and sessile bacteria (filled squares and hatched bars) as described in Materials and Methods. Results are means ± standard deviations of three independent measurements.

thesized by attached cells versus planktonic cells are significantly different (11). Our study suggests that such a genetic reprogramming of gene expression in *E. coli* biofilms results from changes in multiple environmental physicochemical conditions: bacteria within biofilms encounter higher-osmolarity conditions, a lower oxygen supply, and higher cell density. Indeed, the intracellular concentration of potassium ions, which is essentially proportional to the osmolarity of the external medium in the absence of exogenous solutes such as proline or betaine (18), is 1.6-fold higher in attached bacteria than in free-living bacteria (see Results). This magnitude of change is weak but corresponds to that previously published (18; see Fig. 3 of reference 29). According to these studies, our data correspond to external osmolarities of about 220 mosM (corresponding to an intracellular potassium concentration of 223 mM) for free-living bacteria and about 380 mosM (corresponding to an intracellular potassium concentration of 349 mM) for attached cells. The osmolarity of the freshly prepared sterile M63-mannitol medium was 250 mosM (as measured with a Fiske OS/220 osmometer) (data not shown). A bacterium approaching a surface may encounter a gradient of organic and inorganic ions attracted to counterbalance the negative electric charges existing at the solid surface (22). Moreover, the copious amount of appendages present at the cell surface (such as curli [Fig. 2B]) and the various exopolymers excreted by bacteria could concentrate ionic molecules from the bulk phase as the biofilm develops.

Osmoregulated genes, whether they belong to the OmpR regulon (such as *ompC*, *wcaB*, and *f92*) (Table 2) or not (such as *fliC*, and *proU*) (Table 2), respond within biofilms to changes in osmolarity. Fusions up-regulated by high osmolarity (such as *ompC*, *wcaB*, and *proU-lacZ*) were more expressed in sessile cells, whereas fusions down-regulated by high osmolarity (such as *fliC* and *f92-lacZ*) were more expressed in planktonic cells (Fig. 1 and 3). The effect of *ompR234*, the particular allele used in this work, on these responses was investigated for one OmpR-independent osmoregulated gene, *proU*, and for one OmpR-dependent osmoregulated gene, *wcaB*. For these two genes, the same differential expression of free-living bacteria and attached bacteria was observed in biofilms of wild-type *ompR* strains overproducing curli through the overexpression of the activator CsgD (Fig. 4). Thus, an *ompR234* strain is still able to detect osmotic changes and react efficiently (56); the OmpR234 protein is thought to interact more strongly with the regulatory sites of the target genes or with the RNA polymerase. This hypothesis is in good agreement with the higher repression of the *wca* promoter observed with OmpR234 than with OmpR (Table 2).

As the biofilm develops, oxygen consumption and emission of density signals could cause new patterns of gene expression. Using microelectrodes, Costerton and collaborators have shown that microaerophilic conditions were encountered by attached bacteria (11). Here, by using a fusion in *nikA*, a gene which is highly expressed in anaerobiosis and belongs to the FNR regulon (57), limitation of oxygen availability to cells within biofilms and its impact on gene expression were demonstrated (Fig. 5). A 100-fold-higher density was encountered in biofilms of *ompR234 E. coli* K-12 mutants than in the liquid phase. Cell-to-cell signals may therefore be accumulated and be involved in regulation of gene expression. This seems to be the case for *pepT*. This gene was shown to be more highly

TABLE 3. Comparison of osmoregulated fusion expression in attached and free-living bacteria grown under standard conditions or under anaerobiosis

Fusion	Culture conditions[a]	β-Galactosidase sp act (U/mg protein)[b]			
		23 h		45 h	
		Free bacteria	Attached bacteria	Free bacteria	Attached bacteria
f92-lacZ	Standard	16.8 ± 3.6	10.8 ± 0.9	20.0 ± 5.0	11.0 ± 0.4
	Anaerobiosis	ND[c]	ND	15.0 ± 3.7	7.8 ± 0.9
ompC-lacZ	Standard	475 ± 5	1,498 ± 194	454 ± 34	1,497 ± 114
	Anaerobiosis	614 ± 1	1,340 ± 50	644 ± 51	1,530 ± 67
proU-lacZ	Standard	7.9 ± 1.7	21.9 ± 4.9	21.0 ± 1.6	32.5 ± 4.7
	Anaerobiosis	0	5.0 ± 2.4	ND	ND

[a] Biofilm cultures were performed in M63-mannitol medium at 30°C in petri dishes incubated in anaerobic jars (anaerobiosis) or not (standard).
[b] Results are means ± standard deviations of three determinations.
[c] ND, not determined.

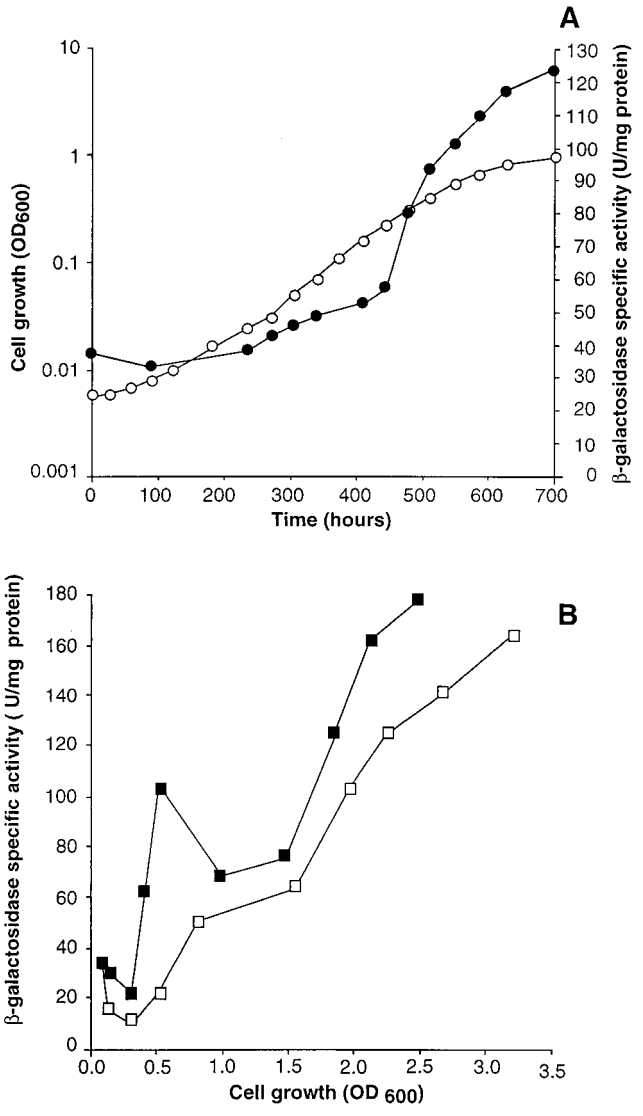

FIG. 7. Effects of cell density on expression of the *pepT-lacZ* fusion. (A) The PHL665 strain was grown in M63-mannitol medium in a shaker at 30°C. Samples were taken at intervals for determination of OD_{600} (open circles) and specific β-galactosidase activity (filled circles). (B) Bacteria were grown in LB medium (open squares) or conditioned LB medium prepared as described in Materials and Methods from a stationary-phase culture of strain DH5α (filled squares). Growth curves in the two media were similar. This experiment is representative of three.

expressed in biofilm cells than in free-living bacteria and to be highly induced in the late exponential growth phase, suggesting its probable regulation by quorum sensing. In *E. coli* K-12 prototroph strains, such as MG1655, two types of autoinducers are produced: the first, encoded by the *luxS* gene (similar to the AI-2 factor produced by *Vibrio harveyi*), is expressed in the mid-exponential phase and is degraded when bacteria enter the stationary phase, whereas the second operates in the stationary phase (3). The first one is not produced by the domesticated laboratory strain DH5α, due to the presence of a frameshift mutation in the *luxS* gene (54). Since the *pepT* gene was induced in the late exponential phase, only the role of the second type of signal molecules was investigated: a conditioned medium, prepared from a stationary-phase culture of DH5α cells, was able to induce the expression of the *pepT-lacZ* fusion at a low cell density. *pepT* could therefore be considered a new *cma* (for conditioned medium activated) gene (3). Previously described *cma* genes were shown to be involved in amino acid metabolism; this fact is in good agreement with the function of tripeptidase T.

Biofilm gene expression patterns appear to be modulated by multiple changing external physicochemical conditions and to involve very complex regulation pathways; several bacterial sensors, many two-component signal transducing systems (such as OmpR-EnvZ), and perhaps some alternative sigma factors (such as AlgU, which is required for alginate gene expression in *P. aeruginosa* [16, 33]), may be suggested to coordinately regulate genes within biofilms. The analysis of additional biofilm-regulated genes isolated in the screen described in this study is in progress. It will allow us to assess the role of physicochemical factors other than osmolarity, oxygen limitation, and cell density in biofilm gene expression in *E. coli* K-12. Further studies of bacterial structures involved in surface sensing and biofilm formation may provide ways to move forward in the search for efficient surface coating methods able to prevent biofilm formation or, at least, to interfere with their inconvenient increased resistance to biocides.

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