

## Nickel Promotes Biofilm Formation by *Escherichia coli* K-12 Strains That Produce Curli<sup>∇</sup>

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**The survival of bacteria exposed to toxic compounds is a multifactorial phenomenon, involving well-known molecular mechanisms of resistance but also less-well-understood mechanisms of tolerance that need to be clarified. In particular, the contribution of biofilm formation to survival in the presence of toxic compounds, such as nickel, was investigated in this study. We found that a subinhibitory concentration of nickel leads *Escherichia coli* bacteria to change their lifestyle, developing biofilm structures rather than growing as free-floating cells. Interestingly, whereas nickel and magnesium both alter the global cell surface charge, only nickel promotes biofilm formation in our system. Genetic evidence indicates that biofilm formation induced by nickel is mediated by the transcriptional induction of the adhesive curli-encoding genes. Biofilm formation induced by nickel does not rely on efflux mechanisms using the RcnA pump, as these require a higher concentration of nickel to be activated. Our results demonstrate that the nickel-induced biofilm formation in *E. coli* is an adaptational process, occurring through a transcriptional effect on genes coding for adherence structures. The biofilm lifestyle is obviously a selective advantage in the presence of nickel, but the means by which it improves bacterial survival needs to be investigated.**

Nickel is not well known to the public, since the major use of this metal is in the preparation of alloys. Nickel alloys are known for their superior resistance to both heat and corrosion. These properties make nickel useful in the chemical industry as well as in food processing (storage tanks, piping, etc.). Nickel alloys are also commonly used in medical devices (pacemakers, orthopedic implants, needles, surgical instruments, etc.). One of the major corrosion products of stainless steel is nickel, and bacteria in the surrounding media encounter these metal ions. Nickel alloys are claimed to ensure that the product or implant remains uncontaminated (26). However, increasing evidence shows that bacteria can adhere to any kind of material to develop biofilms, with detrimental consequences. The development of a biofilm displaying multiresistance to antibiotics, detergents, and sanitizers is a cause of growing concern for hospitals and industrial plants (20, 60).

Although *Escherichia coli* is the best-characterized living microorganism nowadays, current models of biofilm formation come mostly from experimental evidence using *Pseudomonas aeruginosa*, and this phenomenon is described as a multistep process. Reversible physicochemical interactions between bacteria and surfaces allow the initial bacterial attachment in a primary phase. Extracellular polymers, such as fimbriae or capsules, would then allow stable, irreversible molecular and cellular interactions to occur between bacteria and the surfaces. Depending on the extracellular polymers produced, bac-

terial growth results in a mushroom-shaped structure or a flat biofilm (32–34).

Bacterial survival in the presence of toxic compounds is dependent on resistance and tolerance mechanisms. Bacterial resistance is thought to be achieved using five main strategies: (i) efflux systems, (ii) sequestration of the toxic compound, (iii) reduction of bacterial membrane permeability, (iv) enzymatic alteration to a less toxic form, and (v) reduction in the sensitivity of cellular targets (9). These resistance mechanisms allow bacterial growth to occur even at high concentrations of an antibiotic or metal. In contrast to resistance, some cells are able to survive attack by metals or antibiotics without expressing or using resistance mechanisms, but these cells, referred to as persister or dormant cells, are not able to divide (39). Such persister cells were first described in planktonic culture (5) but have been recently characterized in biofilms (24, 25, 54). Poor penetration of antimicrobial agents in the biofilm polymeric matrix and specific metabolic changes in biofilm cells are also thought to be key elements of tolerance. The variety of potential mechanisms implicated in biofilm resistance/tolerance to antimicrobial agents has been recently reviewed (10, 16), but a consensus explanation for the enhanced antimicrobial resistance/tolerance of a biofilm has not yet been demonstrated.

After *Pseudomonas* and *Staphylococcus*, *E. coli* has been the most studied species in terms of biofilms and survival in reaction to metals and antibiotics. Planktonic *E. coli* bacteria can export excess metal by efflux systems, such as RcnA, a nickel and cobalt exporter (51), but no data on the expression of this pump in a biofilm have been published. *E. coli* is a human commensal or pathogenic bacterium that is able to colonize a wide range of habitats, from water to the different organs of its

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

Strain or plasmid	Relevant genotype	Source or reference
<b>Strains</b>		
MG1655	$\lambda^- F^-$	Laboratory collection
PHL818	MG1655 <i>ompR234 malT::Tn10</i>	47
PHL881	<i>E. coli</i> isolated from percutaneous transhepatic catheter	18
PHL1087	PHL818 <i>csgD::uidA-kan</i>	47
PHL1137	PHL818 <i>csgA::uidA-kan</i>	This study
PHL1273	PHL818/p127	This study
PHL1359	PHL818/pMP2444	This study
PHL1370	PHL818/p157	This study
PHL1374	PHL818 <i>rcnA::uidA-kan</i>	This study
PHL1563	PHL818 <i>flu::cm</i>	This study
ZK2692	W3110 $\Delta(\text{argF-lac})$ U169 <i>flu::cm</i>	14
<b>Plasmids</b>		
pCSG4	pUC19 with a 3.5-kb HindIII fragment containing the <i>csg</i> intergenic region; Amp <sup>r</sup>	44
pPROBE-gfp[LVA]	Gene fusion vector, pBBR1 replicon, GFP-destabilized variant LVA; Kan <sup>r</sup>	43
pPROBE-OT'	Gene fusion vector, pBBR1 replicon, GFP reporter gene; Spc <sup>r</sup>	43
p127	pPROBE-gfp[LVA] carrying the <i>gfp</i> [LVA] coding sequence under the control of the <i>csgBA</i> promoter; Kan <sup>r</sup>	This study
p157	pPROBE OT' carrying the <i>gfp</i> coding sequence under the control of the <i>rcnA</i> promoter; Spc <sup>r</sup>	This study
pMP2444	pBBR1MCS-5 containing the enhanced <i>gfp</i> coding sequence under the control of the <i>lac</i> promoter	6

host. This bacterium is able to exist for a long time in the environment, preferentially in biofilms. Lack of data relating to sessile *E. coli* is mainly explained by the difficulty of cultivating biofilms from *E. coli* laboratory strains. Indeed, most of the K-12 strains do not express many adherence factors. Involvement of the curli in biofilm formation on inert surfaces has been discovered by characterizing a curli-expressing adherent mutant derived from the MG1655 laboratory strain (18, 48). Curli are produced by the majority of the enterotoxigenic and enterohemorrhagic *E. coli* strains, including O157:H7, and are regarded as a major virulence factor for pathogenic bacteria (4). The study of the regulation of curli genes in biofilms has led us to propose a model in which they are produced throughout the formation of a biofilm (31, 47). Curli are of particular interest since they are involved in attachment both on eukaryotic surfaces (2, 22) and on abiotic surfaces (glass, sand, plastic, etc.) (7, 38, 58). The genes necessary for curli formation are clustered in two divergently transcribed operons. The *csgBA* operon carries the structural genes. The *csgDEFG* operon encodes the transcriptional activator, CsgD, of the *csgBA* operon and three curli assembly factors. Complex regulatory pathways involving several two-component systems have been shown to control curli gene expression (18, 31, 37).

Numerous studies dealing with the biocide resistance of biofilms highlight the multifactorial aspect of the phenomenon. This work investigates the contribution of the biofilm lifestyle to survival of *E. coli* K-12 strains that produce curli in the presence of the heavy metal nickel. Because this strain is able to form thick biofilms (58), it was used as a model of environmental and medical *E. coli* strains that produce curli (4). Increasing amounts of soluble nickel ions were used to test the early physiological effects of this metal on the adherence and survival of bacteria on polystyrene and stainless steel. Subinhibitory concentrations of nickel promote the formation of very thick biofilms. This biofilm promoter effect does not rely

on the modification of physicochemical cell surface properties by nickel. Surprisingly, this effect also seems to be independent of the efflux pump RcnA. Instead the biofilm promoter effect of nickel results from the drastic upregulation of the curli. We describe here, for the first time, the induction of biofilm formation by a subinhibitory concentration of nickel via the transcriptional activation of curli genes.

## MATERIALS AND METHODS

**Bacterial strains and media.** For this study, we used the *E. coli* K-12 derivative MG1655 *ompR234* (PHL818) and derivatives of this strain (Table 1). This strain produces thick biofilms as a consequence of the expression of curli (47). Bacterial cells were grown in 4× diluted Luria-Bertani broth (LB/4) supplemented with 0.1% mannitol. LB was diluted in order to reduce osmolarity and promote bacterial adherence. Bacteria were grown at 30°C or 37°C. Planktonic growth of the cells was carried out in shaken tubes or Erlenmeyer flasks at 50 rpm or 130 rpm for 24 h. When necessary, ampicillin (100 µg/ml), chloramphenicol (20 µg/ml), kanamycin (50 µg/ml), gentamicin (10 µg/ml), or spectinomycin (100 µg/ml), purchased from Sigma, was added.

**Genetic methods.** The *rcnA::uidA-kan* mutation was transferred from strain ARY023 (51) to PHL818 by phage P1 *vir* transduction, which was carried out as described by Miller (42). Kanamycin-resistant colonies were selected and tested for nickel resistance as described previously (51). The *flu::cm* mutation was transferred from strain ZK2692 (14) to PHL818 by phage P1 *vir* transduction, which was carried out as described above. Chloramphenicol-resistant colonies were selected and tested for nickel resistance as described previously (51). Transformations of *E. coli* cells with plasmids, described in Table 1, were performed as described in reference 11.

**Construction of the *csgB::gfp* and *rcnA::gfp* fusions.** A 757-bp fragment, containing the *csg* intergenic region, was amplified by PCR with plasmid pCSG4 DNA (44) as a template and two primers (5'-TCTAGACATTAACATGATGAAACCCCGC-3' and 5'-GAGCTCCATGTTGTCACCCTGGACCTGGTC-3') overlapping the *csgBA* and *csgDEFG* regulatory regions and containing XbaI and SacI cutting sites, respectively. The PCR fragment was first cloned in pGEM-T (Promega) to make pGEM-T-100/101. The 757-bp XbaI-SacI fragment was cloned into the corresponding sites of the pPROBE-gfp[LVA] vector (43), producing p127 described in Table 1. To construct the *rcnA::gfp* fusion, a 541-bp fragment containing the upstream region of *rcnA* was isolated from plasmid pAR020 (51) after digestion with EcoRI and PvuII. This fragment was inserted

between the EcoRI and SmaI sites of vector pPROBE-OT' (43), resulting in plasmid p157. The plasmids used in this report are listed in Table 1.

**Nickel and magnesium susceptibility testing.** Stock solutions of nickel chloride ( $\text{NiCl}_2$ ; Merck) and magnesium chloride ( $\text{MgCl}_2$ ; Prolabo) were made up to a concentration of 50 mM of the metal cation in ultrapure water (Millipore). The filtered solutions were stored at 4°C. Working solutions (ranging from 100 to 300  $\mu\text{M}$  of the metal cations) were prepared extemporaneously in LB/4. Free-living and sessile cell biomass was estimated by measurements of the optical density at 600 nm ( $\text{OD}_{600}$ ). In each set of experiments, the  $\text{OD}_{600}$  value measured from the culture without nickel or magnesium was taken as the 100% value to normalize the data (arbitrary units [AU]). Growth in the presence of nickel or magnesium is expressed as a percentage of the growth without these metals.

**Fluorescence assay.** The transcriptional activity of the *lac* or *csj* promoter was estimated from measurements of the green fluorescent protein (GFP) fluorescence of cells grown in LB/4 medium containing a range of added nickel concentrations. Fluorescence measurements were made on a Kontron SMF25 fluorimeter with an excitation wavelength of 490 nm and an emission wavelength of 510 nm, after determination of the  $\text{OD}_{600}$  of the cultures. The fluorescence values of three independently grown cultures were averaged and normalized according to the density of the culture. Fluorescence was expressed as relative AU.

**TEM observations.** For transmission electron microscopy (TEM), cell cultures were prepared in flasks containing LB/4 medium supplemented with 0.1% mannitol for 24 h at 30°C and 50 rpm. Cells were carefully washed with 1 mM EDTA in order to remove excess nickel and allowed to sediment at 4°C. Supernatants were carefully eliminated. Five microliters of sedimented cells was carefully recovered and deposited on carbon-coated 200-mesh grids. After fixation with osmium tetroxide, cell staining was performed using 1% phosphotungstic acid and the grids were observed with a Philips CM120 transmission electron microscope.

**$\beta$ -Glucuronidase assay.**  $\beta$ -Glucuronidase activity in toluene-treated samples was measured by spectrophotometrically monitoring the hydrolysis of *p*-nitrophenyl- $\beta$ -D-glucuronide into *p*-nitrophenol at 405 nm (3). Specific activity was measured and expressed as units per milligram of bacterial dry weight, where 1 U corresponds to 1 nmol of product liberated per mg of total bacterial dry weight.

**Static biofilm formation on polystyrene plates.** The wells of 24-well plates or petri dishes were filled with 2 and 20 ml, respectively, of the appropriate medium and inoculated with  $10^6$  cells of an overnight culture. Free-living and sessile bacteria were collected as described in references 18 and 49. Scraping, pipetting up and down, and vortexing for 20 s resulted in disruption of cell clumps, as monitored by microscopy. Free-living and sessile cell biomass was estimated by  $\text{OD}_{600}$  measurements. A minimum of three assays were performed in each experiment. Petri dishes were used to collect a sufficient amount of bacteria to calculate the specific activity when assaying gene fusions in a high-nickel environment.

**Static biofilm formation on stainless steel coupons.** Biofilms were grown on AISI 304 stainless steel (Goodfellow, Cambridge Science Park, United Kingdom). Prior to the biofilm experiments, the solid surfaces were soaked for 10 min at 50°C in a 2% (vol/vol) solution of a commercial surfactant, RBS 35 (Société des Traitements Chimiques de Surface, Lambertsart, France), rinsed for 10 min in Purit deionized water (Purit, France) at 50°C, and then rinsed five times in 500 ml of Purit deionized water at room temperature. The cleaned surfaces were finally sterilized by autoclaving for 20 min at 121°C. Overnight cultures of MG1655 *ompR234* derivatives were diluted to a concentration of  $1 \times 10^6$   $\text{CFU} \cdot \text{ml}^{-1}$ . Twenty milliliters of the bacterial suspension was transferred to petri dishes containing the sterile solid surface of stainless steel. After 2 h of contact, the slides were rinsed with LB/4 to remove the nonadherent bacteria. Adherent cells were finally incubated at 30°C in 20 ml of LB/4 for 24 h.

**Biofilm quantification by laser confocal imaging.** Biofilm quantification was assessed directly on the solid surfaces by confocal microscopy and image analysis. Twenty-four-hour-old biofilms were observed under a SP2 AOBs Leica confocal laser microscope (Leica Microsystems, France, at the MIMA2 microscopic platform). GFP was excited at 488 nm, and the bacterial fluorescence was collected in the range 500 to 600 nm. A Leica  $\times 63/1.4$  N.A. oil immersion objective was used for acquiring images in scanning mode. The overall three-dimensional structures of the biofilms were scanned from the solid surface to the interface with the growth medium, using a step of 1  $\mu\text{m}$ . Quantification of biofilm biovolumes ( $\mu\text{m}^3$ ) was extracted from confocal z-stacks with the PHLIP MATLAB routine, developed by J. Xavier for biofilm structure analysis (<http://www.itqb.unl.pt:1141/~webpages/phlip/phlip-ml/>). Three-dimensional projections were performed with IMARIS software (Bitplane, Zürich, Switzerland).

**Bacterial cell EM.** Prior to the electrophoretic mobility (EM) measurements, bacteria were centrifuged at  $2,000 \times g$  for 10 min and rinsed twice in 5 ml of 15

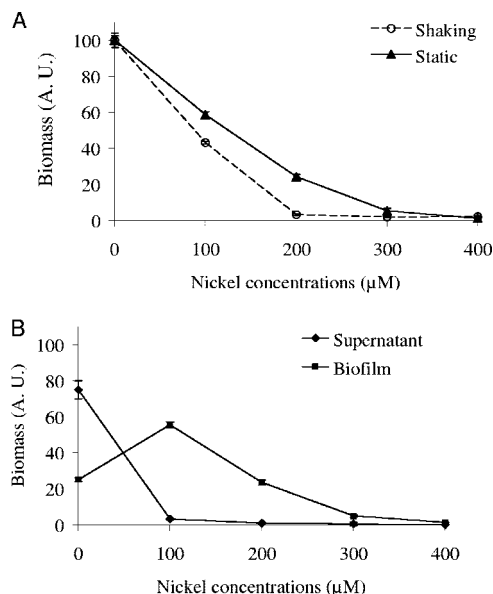


FIG. 1. (A) Inhibition of MG1655 *ompR234* (PHL818) growth by increasing the concentration of nickel. The  $\text{OD}_{600}$  was measured either in cultures that had been shaken (open circles) at 130 rpm or in static cultures (dark triangles), as described in Materials and Methods. The incubation period was 24 h, at 30°C, in LB/4 medium in the presence of increasing nickel concentrations ranging from 0 to 400  $\mu\text{M}$ . (B) Enhancing effect of the low concentration of nickel on biofilm formation on polystyrene. Differential growth of MG1655 *ompR234* (PHL818) in supernatant (dark diamonds) and biofilm (dark squares) fractions was estimated from the  $\text{OD}_{600}$  in petri dish cultures, as described in Materials and Methods. The  $\text{OD}_{600}$  without nickel reached about 1.3 in the supernatant and 0.3 in the biofilm. The addition of supernatant and biofilm fractions resulted in the curve of static growth shown in panel A.

mM sodium chloride at pH 6 to reach a concentration of  $1 \times 10^7$   $\text{CFU} \cdot \text{ml}^{-1}$ . Bacterial suspensions were then incubated for half an hour at room temperature in the presence of 100  $\mu\text{M}$  of nickel chloride, 100  $\mu\text{M}$  of magnesium chloride, or without metal. Electrophoretic measurements were taken in an electric field of 50 V with a Laser Zetameter (CAD Instrumentation, France). Results are based on an automated video analysis of about 300 particles for each measurement. Each experiment was performed in duplicate on two independently prepared cultures.

**Statistical analysis.** The values for each experiment are the mean values of results from three experiments unless otherwise stated. The error bars in figures indicate standard deviation. Analyses of variance were performed with Statgraphics Plus 4.1 (Manugistics, Rockville, MD).

## RESULTS

**Dual effect of nickel on biofilm growth.** As expected, an increasing amount of nickel is toxic for *E. coli* cells cultured under static conditions as well as in shaken cultures (Fig. 1A). Compared to the bacterial growth in the absence of metal, estimated from the  $\text{OD}_{600}$  measurement, 40% of biomass was formed in the presence of 100  $\mu\text{M}$  of nickel in agitated cultures versus 60% in static cultures. Moreover, no growth was observed at 200  $\mu\text{M}$  of nickel in agitated cultures, whereas the inhibitory concentration was 300  $\mu\text{M}$  under static conditions. The toxicity of nickel was therefore significantly less severe in static cultures. A closer insight indicates a complex phenomenon beyond global nickel toxicity in such cultures. Bacteria grown under static conditions were separated into adherent

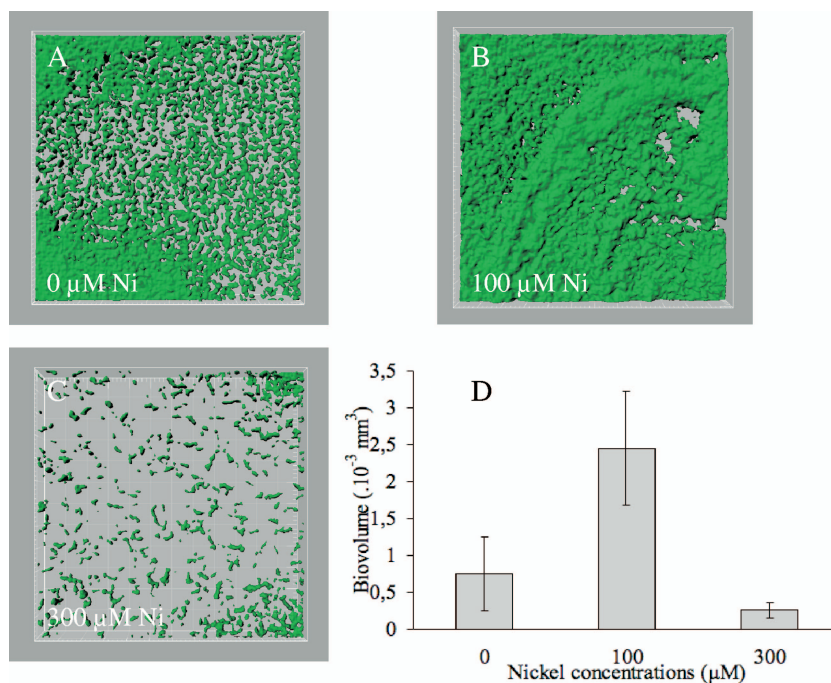


FIG. 2. A low concentration of nickel promotes biofilm formation on stainless steel coupons. Biofilms, formed by the GFP-labeled strain MG1655 *ompR234* (PHL1359), were observed under a confocal microscope after 24 h of growth, as described in Materials and Methods. (A) Uniform biofilm grown without nickel. (B) Irregular three-dimensional structured biofilm grown in the presence of 100  $\mu\text{M}$  of nickel. (C) Microcolonies grown in the presence of 300  $\mu\text{M}$  of nickel. (D) Mean biovolumes calculated from three z-stacks made on each coupon. Variance analysis was performed using Statgraphics (Manugistic, Inc., Rockville, MD). The  $P$  value reflects the probability that the mean of the biovolumes with exposure to nickel is equal to the mean of the biovolumes without exposure ( $P < 0.0001$ ).

and free-floating subpopulations. In this case, an opposite effect of nickel was observed in the two subpopulations (Fig. 1B). The global loss of 40% of the total bacterial population in the presence of 100  $\mu\text{M}$  of nickel in static cultures (Fig. 1A, triangles) results mostly from the disappearance of planktonic cells, and not from a decrease of the biofilm biomass. On the contrary, biofilm biomass increased twofold in the presence of 100  $\mu\text{M}$  of nickel compared to the biofilm grown in the absence of this metal (Fig. 1B). Generation time reached  $1.07 \pm 0.06$  h when the strain was grown without nickel compared to  $1.10 \pm 0.14$  h during growth with 100  $\mu\text{M}$  of nickel (37°C, 130 rpm). This nickel concentration did not alter significantly the growth rate of *E. coli* MG1655 *ompR234* (Mann-Whitney test,  $P < 0.05$ ). We conclude that a subinhibitory concentration of nickel causes the bacteria to develop inside a biofilm rather than growing as free-floating cells.

To quantify this phenomenon by confocal microscopy and to test another abiotic surface, biofilms were grown on steel coupons from *E. coli* MG1655 *ompR234*, expressing a *lac-gfp* fusion from a low-copy plasmid, in the presence of an increasing amount of nickel. Immobilized cells growing on the surface of the coupons were visualized by confocal microscopy. In LB/4 and in the absence of nickel, bacteria developed only a poor biofilm (Fig. 2A), suggesting that LB/4 does not support efficient curli production. However, a thicker and heterogeneous biofilm was formed in the presence of 100  $\mu\text{M}$  of nickel (Fig. 2B). Only scattered colonies were observed at a sublethal concentration of 300  $\mu\text{M}$  nickel (Fig. 2C). The resulting biovolumes were statistically compared using analysis of variance,

and this revealed a significant increase in biovolume observed in the presence of 100  $\mu\text{M}$  of nickel (Fig. 2D;  $P < 0.05$ ). This method indicates a 3.2-fold increase of the biomass of the biofilm grown in the presence of a low concentration of nickel (100  $\mu\text{M}$ ) and confirms the results obtained with  $\text{OD}_{600}$  measurements on polystyrene. To check that the expression of the *lac-gfp* fusion was not affected by nickel addition to the growth medium, fluorescence measurements of agitated cultures supplemented with 0, 100, and 300  $\mu\text{M}$  nickel were performed. A constant value of 1.2 U of fluorescence/ $\text{OD}_{600}$  was routinely obtained under all conditions tested (data not shown).

**Biofilm formation induced by nickel is not related to bacterial surface charge.** The adhesion behavior of microbial cells has been shown to depend on cell surface physicochemical properties and chemical composition. Cations are known to modify the electrical charges of the bacterial surface, often altering the bacterial adhesion (19, 41, 53). To gain information about the global electrical charge of the bacterial cell surface, we assayed the electrophoretic mobility of the cells grown in the absence or presence of metal (57). The presence of nickel in the culture medium decreased the electronegativity of the cell (Fig. 3;  $P < 0.05$ ). To determine if the biofilm enhancer effect of nickel observed could be attributed to a general property of cations, the influence of another cation was assayed under the same culture conditions. The metal magnesium was chosen because of its chemical proximity to nickel. The presence of magnesium in the culture medium decreased the bacterial cell electronegativity to the same extent as nickel (Fig. 3;  $P < 0.05$ ). The presence of magnesium at a concen-

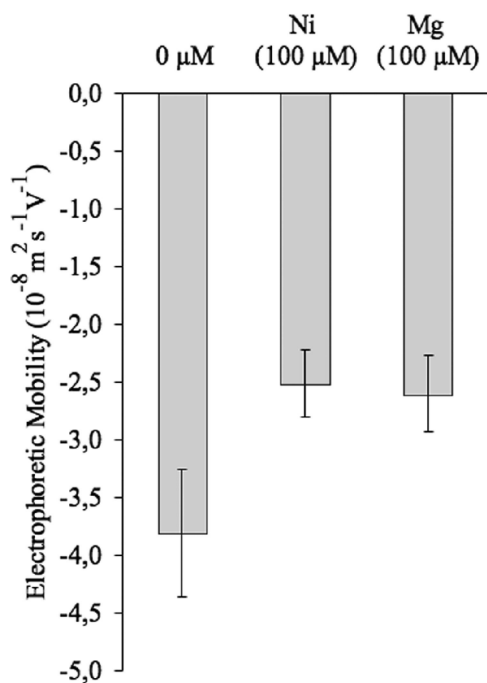


FIG. 3. Electrophoretic mobility of MG1655 *ompR234* (PHL818) in the presence of nickel or magnesium. Similar changes in bacterial surface electronegativity are induced by nickel and magnesium. Electrophoretic mobility of PHL818 was measured after half an hour of contact with 100 μM of nickel or magnesium, as described in Materials and Methods.

tration of 100 μM in the culture medium does not enhance the biofilm formation ( $P > 0.05$ ; data not shown). Therefore, the biofilm formation induced by nickel does not simply rely on a modification of the bacterial surface charge but rather on a physiological effect.

**Biofilm formation induced by nickel does not rely on the nickel-efflux pump RcnA.** Since *E. coli* has been shown to resist high concentrations of nickel by using the inducible efflux pump RcnA (48), we first determined whether the enhancing effect of nickel on *E. coli* biofilms was related to this nickel-resistance mechanism. To test the implication of the Ni/Co efflux pump RcnA in the formation of biofilms in a nickel rich medium, the RcnA-defective MG1655 *ompR234* mutant PHL1374 was constructed by transducing the *rcnA::uidA*-KanR cassette from strain ARY023 (48). The *rcnA* mutation significantly impairs the growth of bacteria in the presence of nickel, whether it is in the biofilm or in the supernatant fractions, as compared to the parental strain (Fig. 4). More surprising for this strain, we confirmed that the presence of 100 μM of nickel in the growth medium promotes biofilm formation, whereas it strongly impairs the growth of free-living cells (compare Fig. 4A and B), as previously observed for the parental strain (Fig. 1 and 4).

The transcriptional level of *rcnA* in the parental background PHL818 was estimated from the activity of an *rcnA-gfp* fusion carried by the low-copy plasmid p157. The *rcnA-gfp* fusion activity in a biofilm is indeed more active at a high concentration of nickel (Fig. 4A), suggesting that the efflux pump RcnA operates mainly in the presence of a high concentration of

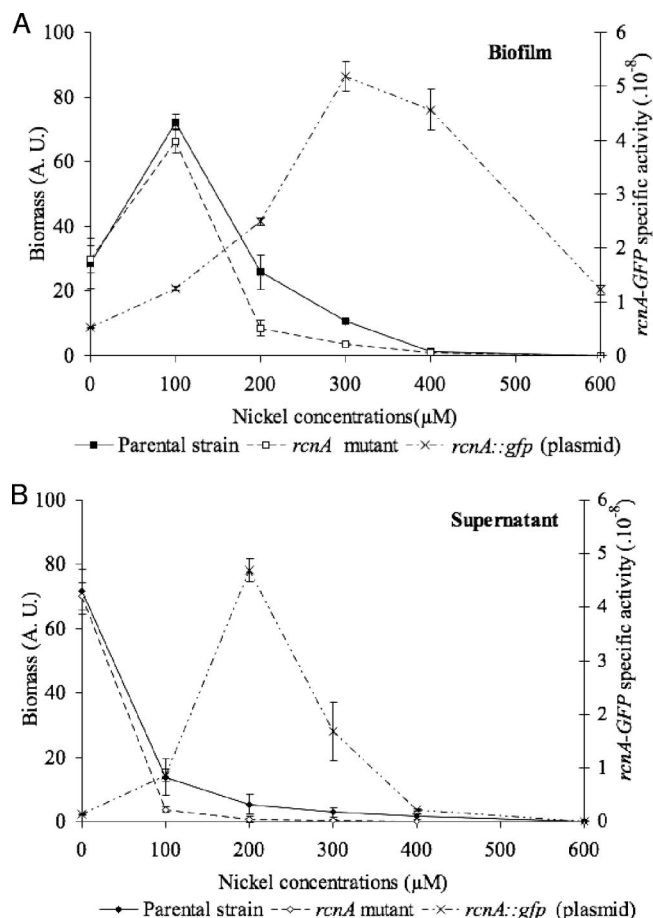


FIG. 4. The nickel promoter effect in MG1655 *ompR234* PHL818 biofilm formation does not rely on the nickel efflux pump RcnA. (A) Biofilm fractions. (B) Planktonic fractions. Growth of MG1655 *ompR234* (PHL818; closed symbols) and its *rcnA* mutant (PHL1374; open symbols) was estimated from the  $OD_{600}$  in supernatant and biofilm fractions in 24-well polystyrene plates. The fluorescence of PHL818 harboring the *rcnA::gfp* fusion (PHL1370) was measured in supernatant and biofilm fractions using a spectrofluorimeter, as described in Materials and Methods. Specific activity for GFP fluorescence (dashed lines) was calculated as follows: (GFP fluorescence – initial fluorescence)/ $OD_{600}$ . If  $OD_{600}$  values of one of the fractions were low (below 0.1 U), assays were repeated in petri dishes to collect larger volumes of culture, leading to a more precise quantification of bacterial growth.

nickel. These results show that biofilm formation induced by a low concentration of nickel does not result from a selective advantage conferred by the presence of the pump. Data obtained from planktonic cultures confirm that high transcription of the efflux pump gene is observed under harsh conditions (less than 20% of cell survival; Fig. 4B).

**Biofilm formation induced by nickel is mediated by curli.** Another possibility to explain biofilm formation induced by nickel is that nickel molecules are able to modify the bacterial physiology in a way that increases the adherence properties of each cell. Antigen 43 (Ag43) is a surface-displayed autotransporter protein able to mediate autoaggregation and biofilm formation of *E. coli*. Mutation of the *flu* gene encoding Ag43 did not affect biofilm formation induced by nickel (Fig. 5). Since curli are one of the main adherence factors in *E. coli*

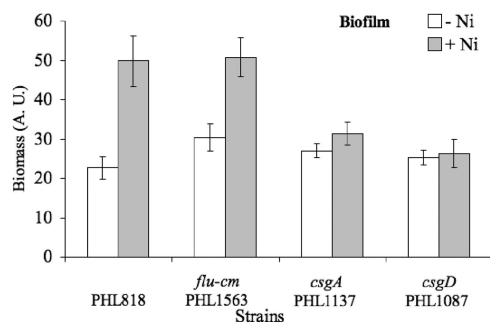


FIG. 5. Involvement of curli in the biofilm formation induced by nickel. PHL818 and PHL818 mutants, defective for Ag43 (PHL1563) or curli production (PHL1087 and PHL1137), were assayed for adherence in LB/4 without (light gray) or with 100  $\mu$ M of nickel (dark gray), at 30°C, in 24-well plates, as described in Materials and Methods. For clarity of the figure, biofilm growth only is shown; growth in the supernatant fraction can be calculated as follows: 100% - biofilm fractions. The values are means for two independent experiments of quadruple cultures; the error bars indicate standard deviations.

strain MG1655 *ompR234* (58), we tested the hypothesis that nickel could mediate biofilm formation induced by nickel by increasing curli production. First, the effect of a subinhibitory concentration of nickel was tested on non-curli-producing strains PHL1137 (*csgA*) and PHL1087 (*csgD*). Biofilm formation induced by nickel was significantly reduced (Fig. 5;  $P < 0.05$ ), indicating that both genes are required to develop thick biofilms. Moreover, Fig. 5 shows that neither Ag43 nor curli are involved in the poor adherence (20%) occurring in LB/4. Poor adherence obtained in plain BL/4 probably results from the production of another adherence structure (exopolysaccharide or adhesin). To measure the transcription activity of the curli promoter, we inserted a reporter gene coding for a GFP with a short half-life (1) under the control of the promoter *csgBA*. This construct was introduced into the MG1655 *ompR234* strain. To avoid problems with the number of copies and stability of the plasmids, the *csgB-gfp*[LVA] fusion was initially inserted as a monocopy at the *trp* locus of the MG1655 *ompR234* chromosome. However, the degree of expression of the fusion remained at the limit of the detection level, irrespective of the culture medium used (data not shown). Thus, the level of transcription of the curli promoter makes it difficult to work with a chromosomal monocopy linked to a reporter gene coding for GFP with a reduced half-life. To overcome this problem, we introduced into MG1655 *ompR234* the *csgB::gfp*[LVA] fusion on a stable plasmid with a low copy number (p127), giving rise to strain PHL1273. To determine if transcriptional induction of curli by nickel occurs during biofilm formation, the level and localization of the *csgB-gfp*[LVA] fusion activity were analyzed using confocal scanning laser microscopy on biofilms grown on a steel coupon, as described in Materials and Methods. For that purpose, bacteria were allowed to develop as a biofilm on stainless steel coupons for 24 h. Nickel was then added to a final concentration of 100  $\mu$ M, and the biofilm was allowed to grow for an additional 3 h. Visualization of the whole biofilm was performed with Syto63 DNA staining (Fig. 6A and C), and GFP expression, driven from the curli promoter, was also assayed (Fig. 6B and D). With the same microscope settings, the bright GFP expression

detected in the presence of nickel (Fig. 6D) was no longer detected in the absence of the metal (Fig. 6B). These results show that supplementation of the growth media with 100  $\mu$ M of nickel quickly overinduces the expression of curli in the biofilm. From this experiment, we conclude that a 3-h contact with nickel was not long enough to modify the structure of the biofilm (compare Fig. 6A and C) but was sufficient to cause a striking increase in curli transcription (compare Fig. 6B and D). Longer exposure to subinhibitory concentrations of nickel results in biofilm thickening (Fig. 2B), very likely as a result of curli production following curli gene transcriptional activation.

**Subinhibitory concentration of nickel activates the expression of *csg* genes.** In a liquid batch culture in LB/4, without nickel, the level of expression of the *csgB-gfp*[LVA] fusion increased slightly at the end of the exponential phase, but only a low level of expression was observed (Fig. 7A). Supplementation with nickel from the beginning of the growth experiment leads to a striking increase in *csgB-gfp*[LVA] expression from entry into the stationary phase (Fig. 7A). The induction of curli production by nickel was confirmed using direct observation by electron microscopy. Strain PHL818, grown in either the absence of nickel or the presence of 100  $\mu$ M nickel chloride, was observed after negative staining. The global observation under both conditions confirmed that cell aggregates are larger and more numerous in the presence of nickel. A close-up of bacterial cells (Fig. 7B) shows clearly that curli are scarce in the absence of nickel, whereas a bushy curli coat is observed when growth occurred with nickel. These microscopic observations are in direct correlation with the *csgA::gfp* assay. This was further confirmed by the use of the *csgA-uidA* fusion, where the presence of 100  $\mu$ M of nickel in the medium leads to a 3.7-fold-increase in *csgB* expression (Fig. 7C;  $P < 0.05$ ). We checked that addition of magnesium at a concentration of 100  $\mu$ M had no effect on the expression of curli genes (data not shown). Since magnesium does not enhance biofilm formation (see above) or *csgB* transcription, we conclude that a low concentration of nickel activates the transcription of *csgBA* genes in a specific manner.

In *E. coli* K-12, the expression of curli genes is restricted to temperatures below 30°C under microaerophilic conditions. To evaluate the impact of nickel on the known environmental regulations of curli, the expression of curli gene fusions in the presence of nickel was tested at 37°C with vigorous shaking. As expected, poor expression was observed in standard medium at 37°C, but a subinhibitory concentration of nickel drastically enhanced the expression of the *csgA-uidA* fusions (Fig. 7C;  $P < 0.05$ ). Similar results were obtained with *csgB-gfp*[LVA] (data not shown).

Adherence assays demonstrate that a functional CsgD protein is required to obtain the biofilm promoter effect (see above). To test if nickel also affects the transcription of this main activator, a *csgD::uidA* fusion was assayed in the presence of nickel at a concentration of 100  $\mu$ M. Figure 7D shows that nickel increased the *csgD* expression to the same extent under favorable (30°C) and unfavorable (37°C) conditions ( $P < 0.05$ ). Taken together, our results show that biofilm formation induced by nickel is mediated by the activation of curli genes from transcriptional activation of the *csgD* activator gene. Moreover, nickel seems to be able to bypass the usual environmental regulation of *csgD* in the PHL818 strain.

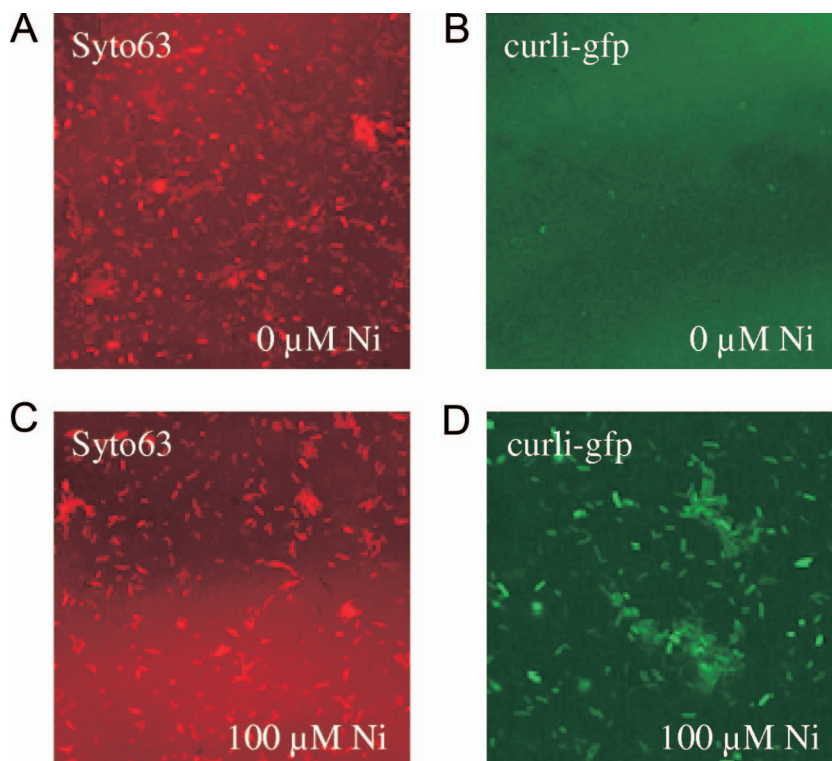


FIG. 6. Induction of curli transcription by nickel in biofilms. Biofilms of the strain PHL1273 were grown for 24 h in LB/4, at 30°C, on stainless steel coupons under static conditions. An additional 3 h of incubation was performed in the absence (A and B) or presence (C and D) of 100  $\mu$ M of nickel. Detection of the living cells was carried out using the fluorescent dye Syto63 (A and C). The GFP fluorescence driven from the curli promoter in the biofilm was compared with (D) and without (B) nickel, with the same microscope settings.

## DISCUSSION

Increased biofilm formation by strain MG1655 *ompR234*, in the presence of a moderate concentration of nickel, was observed on polystyrene and stainless steel coupons. This phenomenon is also observed with the other curli-expressing *E. coli* strain, MC4100 *ompR234* PHL644, and also with the parental MG1655 strain and clinical isolates such as PHL881 (data not shown). Quitting planktonic life to grow a thick biofilm could be a common response of *E. coli* to the presence of a subinhibitory concentration of nickel in the medium.

Distribution of this phenomenon among medical and environmental isolates of *E. coli* needs to be investigated. Among other bacterial species, adhesion of *Desulfovibrio desulfuricans* has been shown to be more extensive on a nickel surface and the growth rate of this bacterium was found to be positively affected by nickel (40). This growth-promoting effect was observed in our system at a lower concentration of nickel (50  $\mu$ M; data not shown). The biofilm formation induced by nickel in *E. coli* occurs at a non-growth-promoting concentration of nickel (100  $\mu$ M) and does not result from a global increase in the bacterial population. Biofilm formation by *E. coli* appears, instead, to be an adaptive and defensive reaction of the bacteria to the presence of nickel. Other biocides have been shown to induce biofilm formation in a wide range of bacteria. Aminoglycoside antibiotics have been shown to induce *E. coli* and *P. aeruginosa* biofilm formation (27). Powerful detergents that disrupt biological membranes, such as sodium dodecyl

sulfate or bile salts, also stimulate biofilm formation in *Pseudomonas aeruginosa* (35), *Vibrio cholerae* (30), and *Bacteroides fragilis* (50). Therefore, we suggest that induction of the biofilm mode of growth could be the early response of bacteria to biocides, allowing the concomitant development of an antibiotic- and metal-resistant phenotype.

Our first hypothesis was that biofilm formation induced by nickel results from enhanced adhesion of bacteria to abiotic surfaces mediated by physicochemical interactions. Bacterial attachment to surfaces is, indeed, the first step in the development of a biofilm. This first step depends on the interactions between the cell surface and abiotic surfaces and is likely to be influenced by the presence of metal in the medium. The cell surface properties of *E. coli* cells were, therefore, characterized by measuring their electrophoretic mobility. As expected, adding nickel to the growth medium strongly modified *E. coli* surface charges. Adding magnesium similarly modified the cell surface charge, but no biofilm increase was then detected. Modification of physicochemical interactions probably plays a key role in early adhesion, but this could not account for biofilm formation induced by nickel. This effect appears to be mediated by the genetic regulation of sticky structures that allow for subsequent biofilm development.

The second hypothesis was tested by assaying several mutants involved in production of the adherence factors. We found that the mutations affecting curli production abolish nickel-induced biofilm formation. These results indicate

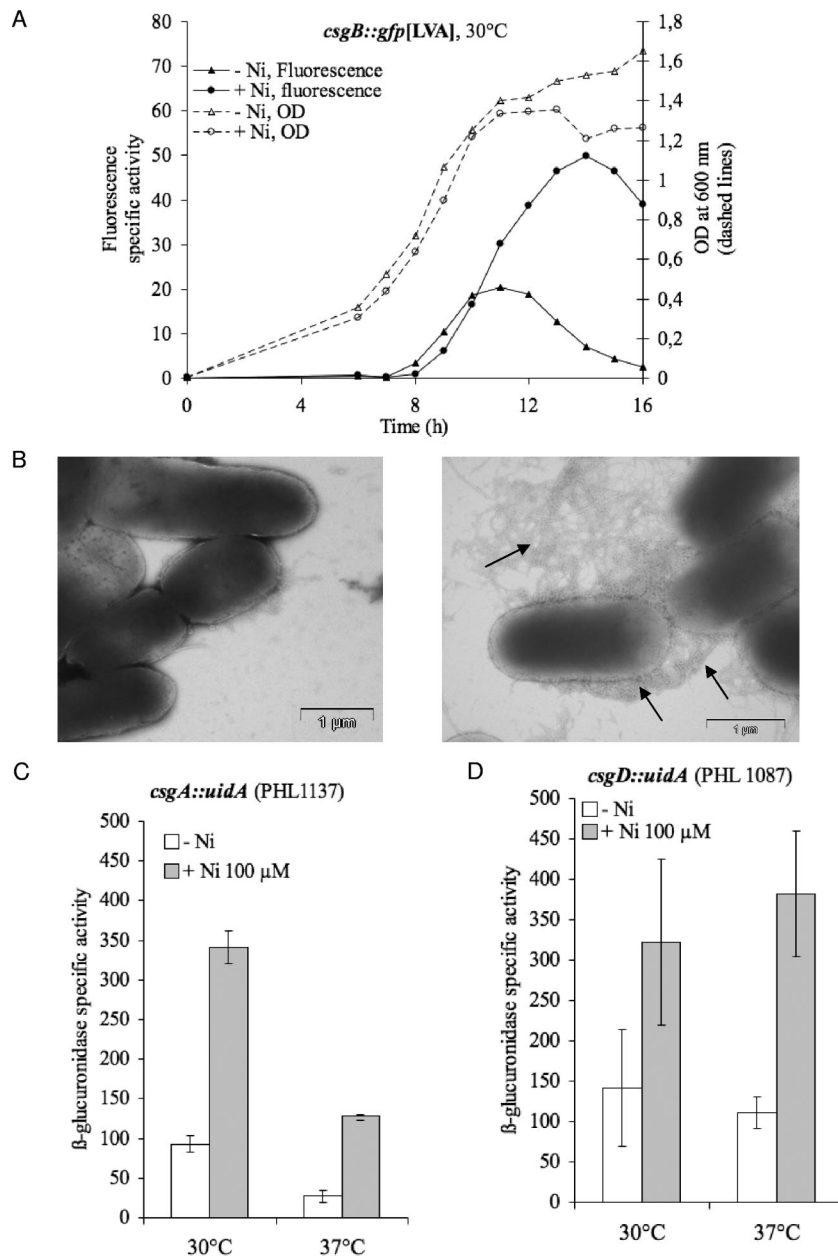


FIG. 7. Overinduction of curli transcription by nickel in batch culture. (A) *csgB-gfp[LVA]* expression under conditions favorable for expression of curli. Planktonic cultures of PHL1273 were grown at 30°C in the presence (open triangles) or absence (open circles) of 100 μM of nickel. Bacterial growth was estimated from the OD<sub>600</sub> (dashed lines). Specific activity for GFP fluorescence (solid lines) was calculated as follows: (GFP fluorescence – initial fluorescence)/OD<sub>600</sub>. Three independent experiments were performed. Results of a typical experiment are presented. (B) TEM observation of the induction of curli production by nickel. Strain PHL818 was grown in the absence (left panel) or presence (right panel) of 100 μM NiCl<sub>2</sub>. The arrows point to curli structure. (C) *csgA::uidA* expression. The expression level of the fusion *csgA::uidA* was compared in the late exponential phase under conditions favorable for curli expression (30°C; left) and conditions unfavorable for curli expression (37°C; right). Strain PHL1137 was grown in planktonic culture in standard medium (white bars) or in the presence of 100 μM of nickel (dark bars). Specific β-glucuronidase activity was calculated as described in Materials and Methods. The values are means for triplicate cultures; the error bars indicate standard deviations. (D) *csgD::uidA* expression. Strain PHL1087 was grown in standard medium (white bars) or in the presence of 100 μM of nickel (dark bars). Other conditions are as described in the legend to panel C.

that curli genes are required to promote the biofilm formation when bacteria encounter subinhibitory concentrations of nickel in the surrounding medium. The transcription of both curli operons was strikingly increased by subinhibitory concentrations of nickel at 30°C and 37°C. Upregulation of

curli genes appears to be the key factor in the biofilm formation induced by nickel. Moreover, the presence of nickel in the surrounding media is able to bypass the strict thermoregulation of curli in K-12 strains. This result shows the high reactivity of the curli promoter to environmental



changes, but the mechanism of nickel action has not yet been elucidated. As for many virulence factors, curli production is heavily regulated at the transcriptional level by environmental factors, such as temperature, osmolarity, oxygen concentration of the medium, pH, the presence of ethanol, and certain nutrient deficiencies (nitrogen, phosphate, and iron [reviewed in reference 37]). Our work shows, for the first time, the reactivity of curli genes to nickel.

Beyond its role as activator of the structural *csgBA* operon, CsgD also regulates genes involved in the synthesis of cell surface structures (cellulose, O antigen, and the determinant for biofilm formation), transport, and metabolism (reviewed in reference 23). Increased transcription of the *csgD* gene potentially results in significant changes in cell physiology associated with the transition from planktonic cells to biofilm. We found that nickel affects the central regulatory knot *csgD*, but the molecular mechanism of this activation still needs to be investigated. A number of regulators are known to affect, positively or negatively, the transcription of the *csgD* promoter (CpxR, Crl, H-NS, MlrA, OmpR, RpoS, and RcsB [reviewed in reference 37]), but the molecular mechanism of the *csgD* promoter activation by nickel remains to be elucidated. It is noteworthy that CpxA/CpxR, which negatively regulates the *csgD* promoter (31), belongs to the copper stimulon (61). This transduction system could also be involved in nickel sensing. We found that the adherence properties of *cpxR* mutants in the presence of a subinhibitory concentration of nickel were not significantly affected. Moreover, no significant effect on transcription of the *csgA::gfp* fusion in mutant strains could be detected (data not shown). The curli are, therefore, induced by nickel but not in a CpxAR-dependent manner. MlrA is a transcriptional activator of the *csgD* promoter which shares similarities with a regulatory protein of the MerR family (MerR-like regulator A) (8). MerR represents one of the five metal sensor families able to bind metal ions and allosterically regulate DNA binding and transcription of target operons (45). However, neither MlrA nor the two nickel-sensing dedicated regulators in *E. coli*, namely NikR (17) and RcnR (36), were shown to be involved in the induction of *csgB* or *csgD* by nickel (our personal data). Induction of biofilm formation by sodium dodecyl sulfate and aminoglycosides was shown to involve cyclic di-GMP (c-di-GMP) signaling (27, 35). This bacterial second messenger controls lifestyle choices in bacteria, such as a commitment to sessile life or virulence (12), and it is antagonistically controlled by diguanylate cyclases (GGDEF proteins) and phosphodiesterases (EAL proteins). Recent studies point to *csgD* as a major target for regulation by c-di-GMP. Several of these c-di-GMP control modules (YdaM/YclR, YegE/YhjH, and YaiC/YoaD) are indeed involved in curli regulatory cascades, with CsgD representing the major signal integration node (7, 46, 59). c-di-GMP signaling could, therefore, mediate biofilm induction by nickel as a global effect of stress on *E. coli* bacterial cells.

A similar effect of nickel on another adherence factor has been recently observed in pathogenic *E. coli*. Low concentrations of nickel were shown to increase the transcription of the bundle-forming pilus (BFP) structural gene (*bfpA*) in enteropathogenic *E. coli*. This effect was specific to nickel, as compared with copper and zinc (13). Nickel influences the tran-

scription of curli and BFP genes, which are both involved in the adherence and virulence of *E. coli*. A global increase of adherence, via the transcriptional regulatory mechanisms of the adherence structure, could be an early response of *E. coli* to nickel and more generally to metals. However, although the accumulation of copper in the *cueO* mutant results in cell aggregation correlated with an increased expression of cell surface adhesion Ag43 and curli (55), a strong inhibition of enteropathogenic *E. coli* (EPEC) adherence factors, such as BFP, EspA, and intimin, was observed in the presence of zinc (13). Metals are clearly involved in adherence, but the effect on adherence structures appears to be metal specific. Other biocides are involved in the transcriptional control of adherence structures. For example, bile salts can cause fimbrial overproduction and increase *E. coli* adhesion to human epithelial cells (15). The involvement of biocides in adherence may therefore be considered as a general phenomenon.

An important question to consider is why bacteria begin to grow inside a biofilm in the presence of a subinhibitory concentration of nickel. Cells in a biofilm could benefit from protection against harsh conditions as a result of higher tolerance mechanisms. In *E. coli*, only one nickel efflux system (RcnA) has been identified (51) and our results show that the efflux pump is not required for the formation of a biofilm in the presence of 100  $\mu$ M of nickel. This efflux pump RcnA seems to be used only as an emergency system and mainly operates at higher nickel concentrations (Fig. 4). Other mechanisms of defense could be provided intrinsically by an *E. coli* biofilm. Curli are thought to enhance resistance to antibacterial agents, such as chlorine or quaternary ammonium sanitizer (52, 56). The amyloid property of curli supports the idea of them having a protective role (21). Formation of biofilm by a strain expressing curli may confer resistance to heavy metals by retarding metal diffusion (28, 29). Indeed, tolerance of *E. coli* biofilms to metals has been demonstrated to be time dependent (25).

Nickel tolerance of biofilms results most probably from the conjunction of several mechanisms that act in a time-dependent manner. We propose that increased adherence occurs as an early cell adaptation to subinhibitory concentrations of nickel and that increased tolerance inherent to the biofilm mode of growth occurs as a consequence. Special attention must therefore be given to the effect of a low concentration of nickel, and more generally of biocides, in the environment or in biomaterials, to avoid biofilm development.

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