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# Biofilm formation by *Escherichia coli* is stimulated by synergistic interactions and co-adhesion mechanisms with adherence-proficient bacteria

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**Abstract**

Laboratory strains of *Escherichia coli* do not show significant ability to attach to solid surfaces and to form biofilms. We compared the adhesion properties of the *E. coli* PHL565 laboratory strain to eight environmental *E. coli* isolates: only four isolates displayed adhesion properties to glass significantly higher than PHL565. The ability of the adhesion-proficient isolates to attach to glass tubes strongly correlated with their ability to express curli (thin aggregative fimbriae), thus suggesting that curli are a common adhesion determinant in environmental strains. Despite its inability to attach to solid surfaces, growth of *E. coli* PHL565 in mixed cultures with *Pseudomonas putida* MT2 resulted in co-adhesion and in formation of a mixed *E. coli*/*P. putida* biofilm, which was able to colonize glass surfaces with dramatic efficiency compared to *P. putida* alone. *E. coli*/*P. putida* interactions stimulate initial adhesion to glass, and the presence of both bacterial species in the mature biofilm was confirmed by quantitative PCR. In contrast, no synergistic biofilm formation was observed in mixed cultures of *E. coli* with the Gram-positive bacterium *Staphylococcus epidermidis*. Interestingly, *E. coli* PHL565 also stimulated biofilm formation by bacterial communities isolated from drinking water distribution systems. Our results strongly suggest that co-adhesion and synergistic interaction with biofilm-forming species might represent an important mechanism, and a possible alternative strategy to production of adhesion determinants, for persistence and propagation of *E. coli* in the environment.


*Keywords:* Biofilms; Adhesion; Curli; Co-adhesion; Drinking water

## 1. Introduction

In natural environments, bacteria are often found as sessile communities, usually referred to as biofilms. Biofilms can colonize nearly all types of materials and develop on basically every surface in any water-rich environments. They are formed by the association of different microbial species, both prokaryotic and eukaryotic [7,12,33]. A striking feature of many biofilms is the extensive production of an extracellular matrix, which is mainly composed of complex polysaccharides and proteins, although a high content of extracellular DNA has also been reported [9,36]. This extensive production of extracellular polymeric structure (EPS) also takes place when biofilm-forming bacteria grow in natural environments where nutrient concentrations are growth-limiting, despite the high energy consumption it requires, suggesting that growth as a biofilm confers important advantages upon the microorganisms. Indeed, biofilms are more resistant to environmental stress such as dehydration and oxidative stress [1,5,12], and to treatment with biocides or detergents, and they show high levels of tolerance to prolonged antibiotic therapy in human and veterinary infections [16].

In addition to playing a part in bacterial infections, biofilms are a health hazard when present in drinking water distribution

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systems. Release of cell clusters from sloughing of biofilms attached to drinking water pipes may result in the periodical release of bacteria in drinking water.

The enterobacterium *Escherichia coli* is universally utilized as a main indicator microorganism to assess the quality of drinking water, because of its ubiquity in fecal-contaminated water, the relative facility of isolation and identification, and its inability to multiply efficiently in the environment [11,32]. Several strains of *E. coli*, either pathogenic or of environmental origin, are able to colonize surfaces through production of adhesion determinants and develop as a biofilm, which could result in longer persistence in the environment and in possible reiterated contamination of drinking water distribution systems. *E. coli* strains can produce a variety of different determinants for attachment to solid surfaces and/or biofilm formation [24,26,34], such as curli [14], type I pili [30], flagella [25], conjugative pili [13] and extracellular polysaccharides such as colanic acid and cellulose [8,26,28]. However, expression of adhesion determinants such as curli remains cryptic in many laboratory strains, despite the presence of functional corresponding genes on the chromosome, which results in lack of ability to attach to solid surfaces. In this report, we compared adhesion properties of the *E. coli* PHL565 laboratory strain to eight *E. coli* strains isolated from reservoir waters. Both adherent and non-adherent phenotypes are represented among environmental isolates; among adhering strains, curli fibers seem to be an important adhesion determinant. However, non-adhering *E. coli* strains can successfully establish themselves in mixed biofilms through co-adhesion mechanisms with adhesion-proficient bacteria.

## 2. Materials and methods

### 2.1. Bacterial isolation and growth

Bacterial strains used in this work were: *Staphylococcus epidermidis* ATCC-155 (American Type Culture Collection), *Pseudomonas putida* MT2 (ATCC-33015) and *E. coli* PHL565 [35]. PHL565 is a K-12 strain equivalent to MG1655; however, we will refer to it as PHL565, since MG1655 strains from different sources can carry mutations responsible for different biochemical properties (Landini, unpublished observations). When not otherwise stated, bacteria were grown in M9sup medium (M9 salts supplemented with 0.5% glucose and 5% LB medium) at 28 °C. For mixed cultures, bacteria were grown separately overnight in M9sup, and diluted by a factor ranging from 1:100 to 1:500, so to achieve a 1:1 ratio between the colony forming units (CFUs) of different bacterial species, as determined by previous plate counting on overnight cultures. Selective plating of mixed cultures after overnight growth showed ratios between different microorganisms close to 1:1 (data not shown). Biofilm formation was determined as described below. Environmental isolates of *E. coli* were collected in drinking water reservoirs in the northern part of Switzerland, at different times and in different locations. Water samples were plated onto ECD-MUC plates, a specific medium for *E. coli*. The isolates were identified as *E. coli* using the API20E system (Bio-Merieux). Out of roughly 20 isolates, we chose eight differing in physiological properties, as determined by growth on various carbon sources (BIOLOG test), the ability to produce either colicins or siderophores, hemolytic activity and sensitivity to antibiotics (data not shown). Bacterial consortia from drinking water catchment areas were isolated in Sneek, Spannenburg and Leeuwarden (The Netherlands), as follows: biofilm sampling devices were placed along the distribution system at various distances, from the origin of a water-winning location. Approximately 30 circular rubber rings were placed in the sampling devices, which were collected at time intervals of approximately ten days over a period of five weeks. Bacteria attached to the rings were resuspended in sterile water and biological activity was tested by determination of ATP presence (reflecting all biological activity); the samples were plated on different media to determine CFUs at both 22 and 37 °C (ranging from 1 to $3 \times 10^2$ after ten-day incubation). We checked for the presence of the bacterial groups enterococci, clostridia, *Legionella* and *Pseudomonas*, as well as of *E. coli*, by plating on selective media. None of these bacterial species could be detected (data not shown).

### 2.2. Adhesion and initial attachment assays

Surface attachment assays were performed using the crystal violet method described in [27], except that glass tubes were used instead of microtiter plates. Briefly, 2 ml cultures were grown overnight in 12-mm diameter glass tubes in M9sup medium. The liquid medium was removed and the attached cells washed with sterile PBS (pH 7.0). The bacterial biofilm was visualized by staining with 1% crystal violet for 20 min at room temperature, followed by rinsing with water and air drying. Quantification of biofilm was achieved by dissolving crystal violet in 3 ml 95% ethanol followed by spectrophotometrical determination at $OD_{600\,nm}$. The value was divided by the $OD_{600\,nm}$ of the corresponding planktonic culture to adjust for total bacterial growth. The $OD_{600\,nm}$ of liquid cultures was not severely affected by the degree of biofilm formation, suggesting that only a relatively small number of cells attach to the surface of microtiter plates in the conditions tested. For quantitative PCR analysis (see below) the cells attached to the glass tubes were mechanically scraped and resuspended in sterile PBS buffer (1 ml). Initial adhesion of bacterial cells to sand-filled columns was performed as previously described [20,31]. Cells were grown to stationary phase in M9sup medium ($OD_{600\,nm} > 2.0$), washed with phosphate buffer saline pH 7.0 (PBS) and resuspended in PBS (30 ml) at an $OD_{280\,nm}$ between 0.8 and 1.0 (corresponding to roughly $OD_{600\,nm} = 0.2$), corresponding to circa $5 \times 10^7$ CFU/ml. The suspension was loaded at the flow rate of 0.5 ml/min onto a 12-cm column filled with 9 g of pure sea sand (Fluka, Switzerland), pre-equilibrated in PBS. For mixed culture experiments, bacterial species were mixed at either a 1:1 or a 1:3 ratio (for experiments with *S. epidermidis*) at an $OD_{600\,nm} = 0.25$ and grown to stationary phase. The ratio between different bacterial species in suspensions loaded onto the sand columns was always very close to 1, as determined by selective plating (data not shown).

### 2.3. Measurement of curli expression

To test for curli production, the *E. coli* strains were spotted on Congo red plates (1% casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$, 2% agar, 40 µg/ml Congo red), and grown overnight at 28 °C. Curli-producing strains are stained intensely by Congo red and grow as dark red colonies [14]. To verify that Congo red staining was indeed due to curli production, we transformed the *E. coli* strains with pPL423, a plasmid carrying the *lacZ* gene under the control of the *csgB* promoter, which directs expression of the *csgB* and *csgA* genes, encoding the curli functional subunits. The plasmid, in addition to the ColE1 origin of replication and the ampicillin resistance *bla* gene, carries the *csgB* promoter region, obtained by PCR amplification, into the *Bam*HI and *Eco*RI sites of the pMV120 plasmid [19] to produce pPL423. The primers used for amplification of the *csgB* promoter region were csgBfw (5′-AATGGATCCCAGCGTATTTACGTGGG-3′) and csgBrev (5′-AATGAATTCTGCAATCCCAGGCGCACC-3′) (restriction endonuclease sites are underlined), annealing respectively between −195 and −178 base pairs and between +40 and +57 base pairs, relative to the ATG start codon of the *csgB* gene. Production of β-galactosidase was tested by plating the different bacterial strains on M9sup agar plates supplemented with 40 µg/ml X-gal. The plates were incubated for 24 h at 28 °C. Expression of β-galactosidase, the product of the *lacZ* gene placed under the control of the *csgB* promoter, results in a blue coloration on plates supplemented with X-gal. Inactivation of the *csgA* gene was carried out by phage P1-mediated transduction.

### 2.4. Quantitative PCR and fluorescent in situ hybridization (FISH) analysis

We determined bacterial concentrations in mixed cultures using quantitative real-time PCR (Q-PCR) with Sybr Green I DNA staining (Applied Biosystems) [2,21]. The DNA template for Q-PCR reactions was obtained by adding 10 µl of lysis buffer (10% Triton X-100, 5% Tween 20, 10 mM Tris–HCl, 1 mM EDTA) to 100 µl of bacterial culture and heating 12 min at 100 °C. For biofilm analysis, attached cells were removed by scraping and vigorous pipetting in M9sup medium. The cell debris was removed by centrifugation (5 min at 14 000 rpm in a table centrifuge) and 1 µl of supernatant was used as template for the PCR. For detection of *E. coli*, two primers targeting the *uidA* gene [3] were adapted for Q-PCR by shorting them with a few nucleotides resulting in the following primers: uidAfw (TGGTGATTACCGACGAAAAC) and uidArev (GCGTGGTTACAGTCTTGC). The two primers yielded a PCR product of 147 base pairs. For *P. putida*, primers targeted at the *oprL* gene of *P. aeruginosa* [10] were adapted on the bases of the *P. putida oprL* gene producing a 498-bp fragment. Primer sequences were: oprLfw (ATGGAAATGCTGAAGTTTGGT) and oprLrev (ACTTACGCAGTTCTACGC).

The reaction protocol for both sets of primers was as follows: 10 s at 95 °C, 20 s at 60 °C (annealing temperature), and 20 s at 72 °C, for 45 cycles. All primers were used at a final concentration of 0.9 µM. Q-PCR Calibration curves performed on pure cultures of either *E. coli* or *P. putida* showed linear correlation down to less than $10^2$ CFU, as determined by parallel plate counting on L agar (data not shown). Bacteria were grown at 30 °C for 24 h. The correlation between CFU and PCR cycles is linear over bacterial concentrations ranging from $10^1$ to $10^6$ for *P. putida* and from $10^2$ to $10^7$ for *E. coli*. After 33 cycles, non-specific signals became detectable with the *E. coli uidA* primers, possibly due to low-level contamination of the Taq DNA polymerase enzyme with *E. coli* DNA. FISH analysis of *P. putida/E. coli* mixed biofilms and of microbial consortia isolated from drinking water distribution systems were performed as described in [22].

## 3. Results

### 3.1. Adhesion properties of environmental isolates of E. coli

We tested the ability of eight environmental isolates of *E. coli* to attach to the glass walls of 12-ml culture tubes. The adhesion properties of the environmental strains were compared to the adhesion-deficient PHL565 laboratory strain and to its biofilm-forming, curli-producing PHL628 mutant derivative [27]. Adhesion experiments were carried out in M9sup medium (described in Section 2) at either 28 or 37 °C. As shown in Fig. 1, four strains (WK1, WK2, WK4, and WK5), were proficient in biofilm formation on glass tubes; one strain (WK8) showed increased ability to grow on a glass surface compared to PHL565, although to a lesser extent than other isolates. Finally, three isolates (WK3, WK6, WK7) showed little or no difference from PHL565, i.e., they were unable to colonize the glass tube. Interestingly, the environmental isolates were only able to adhere to glass when grown at 28 °C; growth at 37 °C resulted in a significant decrease in biofilm formation for most strains, with the exception of WK7 (Fig. 1). Temperature dependence of biofilm formation suggests that the main determinants in the adhesion-proficient strains could be the curli fibers, which are only expressed at 28 °C, but not at 37 °C. Curli (also known as thin aggregative fimbriae in *Salmonella*) are proteinaceous extracellular structures involved in adherence to solid surfaces and in cell–cell aggregation [14,26]. Maxi-

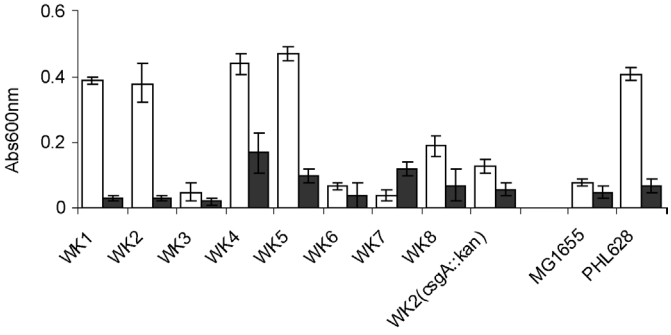


Fig. 1. Biofilm formation on glass tubes by different *E. coli* strains, determined by the crystal violet staining method described in the text. *E. coli* strains were grown overnight in M9sup medium at either 28 °C (white bars) or 37 °C (black bars). Data are the average of three independent experiments.

Table 1
Curli production and *csgB* expression in *E. coli* isolates

| Strain | Phenotype on Congo red plates | Phenotype on X-gal plates after transformation with pPL423 (P*csgB*::*lacZ*) |
|---|---|---|
| *E. coli* PHL565 | White* | White |
| *E. coli* PHL628 | Red | Dark blue |
| *E. coli* WK1 | Red | Dark blue |
| *E. coli* WK2 | Red | Dark blue |
| *E. coli* WK2 (*csgA*::*kan*) | White* | Dark blue |
| *E. coli* WK3 | White* | White |
| *E. coli* WK4 | Red | Dark blue |
| *E. coli* WK5 | Red | Dark blue |
| *E. coli* WK6 | White* | White |
| *E. coli* WK7 | White* | White |
| *E. coli* WK8 | Pink | Light blue |

* White corresponds to a pale pink coloring, clearly distinguishable from the red phenotype of the curli-producing PHL628 strain, and probably due to non-specific binding of the Congo red dye to the cell surface.

mal expression of the curli-encoding *csgBA* genes occurs in low salt media at temperatures lower than 30 °C [4,27,28]. Indeed, the PHL628 strain, an otherwise isogenic mutant of PHL565 in which expression of curli and cellulose is stimulated by a mutation in the OmpR regulatory protein, shows very similar adhesion properties as the more adhering environmental strains (WK1, WK2, WK4, and WK5) as well as the same temperature dependence (Fig. 1). Curli expression in the adhering strains was confirmed by binding of Congo red (a dye that binds amyloid proteins such as curli [14]) on agar plates and by expression of β-galactosidase from a *csgB*::*lacZ* fusion carried by the pPL423 plasmid. Congo red binding and β-galactosidase expression from the P*csgB*::*lacZ* fusion strongly correlated with curli expression and ability to form biofilm on a glass surface (Table 1). To further investigate whether curli expression is indeed responsible for adhesion properties of the *E. coli* environmental isolates, we attempted to inactivate the *csgA* gene, encoding the main curli subunit, by P1-mediated transduction of a *csgA*::*kan* allele from the PHL856 strain [27]. However, almost all environmental isolates were either resistant to phage P1 or already resistant to kanamycin, or both, with the sole exception of WK2, which could be transduced successfully. The so obtained WK9 strain (WK2 *csgA*::*kan*) displayed significant reduction in its ability to attach to glass compared to its parental strain (Fig. 1), and was only faintly colored by Congo red, as expected by lack of curli production.

### 3.2. Co-adhesion experiments

The results of the previous experiments showed that not only the PHL565 laboratory strain but also several environmental isolates lack the ability to attach efficiently to glass and to form biofilm. However, non-adhering bacteria can interact with other microorganisms present in the environment or be recruited by microbial communities to form mixed biofilms [18]. Thus, we tested the possibility that *E. coli* PHL565 might be able to attach to glass in the presence of adhesion-proficient bacteria. Since species belonging to the *Pseudomonas* and *Staphylococcus* genera have been described as forming biofilm very efficiently [12], we tested attachment to glass by PHL565 in the presence of *P. putida* MT2 and *S. epidermidis* ATCC155 laboratory strains. However, only *P. putida* was able to form very thin biofilms on glass tubes in the growth conditions used (Fig. 2), suggesting that, like *E. coli* PHL565, these laboratory strains were also somehow impaired in adhesion to solid surfaces. However, build-up of thin biofilms by *P. putida* and *S. epidermidis* on glass tubes could be measured by crystal violet staining (Fig. 2B).

Interestingly, whilst neither bacterium showed strong adhesion properties, mixed cultures of *E. coli* PHL565 and *P. putida* MT2 displayed a dramatic increase in biofilm formation compared to their pure culture counterparts (Figs. 2A, 2B). In contrast, mixed *S. epidermidis–E. coli*, as well as *S. epidermidis–P. putida*, cultures, did not show any significant increase in colonization of glass tubes (Figs. 2A, 2B), and addition of *S. epidermidis* to *E. coli–P. putida* mixed cultures did not significantly affect biofilm formation (Fig. 2A, sample 7). These experiments were performed so as to achieve a roughly 1:1 ratio between bacterial species in overnight cultures (see also Section 2). To determine the amount of each bacterium in mixed biofilms, we performed quantitative PCR (Q-PCR) analysis of the biofilm. This method was preferred to plating on selective media in order to avoid imprecise CFU determinations due to plating of clumped cells, to possible cell lysis during resuspension of biofilm and poorer growth on some selective media. Q-PCR analysis of biofilms formed by mixed *E. coli* PHL565–*P. putida* MT2 cultures revealed that both species are present in the biofilm at roughly the same concentrations: *E. coli* was present at $3.2 \times 10^5$ CFU in the resuspended biofilm, with a 2.5-log (i.e., 300-fold) increase in the number of surface-attached cells compared to pure *E. coli* cultures (Figs. 2A and 2B). The presence of PHL565, in turn, stimulated attachment by *P. putida* by about 20-fold (1.35-log increase; Fig. 2C). Both *E. coli* PHL565 and *P. putida* MT2, when grown in mixed cultures, were present in the liquid medium at roughly $5 \times 10^8$ CFU/ml, as determined by Q-PCR on diluted samples, while pure cultures reached $10^9$ CFU/ml (data not shown).

Very similar results were obtained when *P. putida* was grown with either the WK2 (adherent) or WK3 (non-adherent) *E. coli* environmental isolates; in both cases, presence of the *E. coli* strain resulted in formation of a mixed biofilm, more efficient in colonization of the glass surface than either strain alone (data not shown).

In order to gather more detailed information on co-adhesion mechanisms, we performed FISH experiments on glass surfaces colonized by *P. putida* and *E. coli*. As shown in Fig. 3, both *E. coli* and *P. putida* cells from mixed cultures are detectable, in roughly equal numbers, already at an early stage on the glass surface, thus suggesting that synergistic interaction between the two species takes place at a very early stage in biofilm formation.

In order to confirm the results of FISH observations, we also tested adhesion of either pure or mixed *E. coli/P. putida* bacterial cultures to sand columns. This method specifically measures

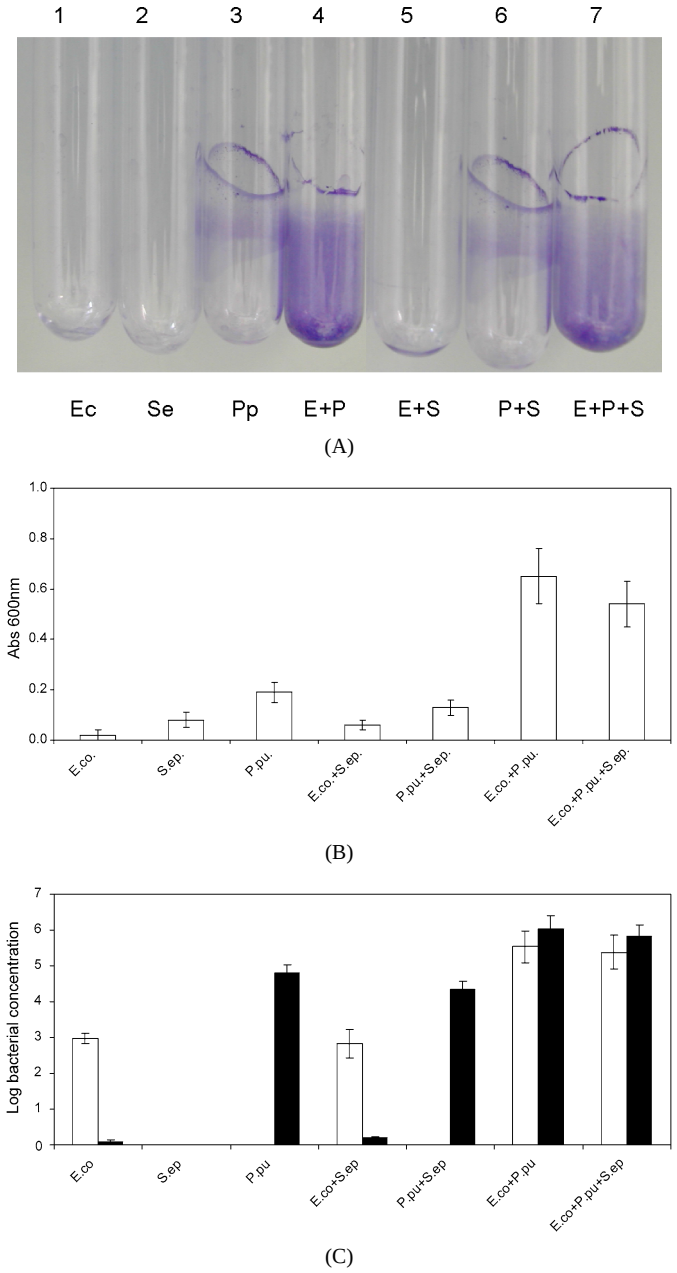


Fig. 2. Biofilm formation on glass tubes by pure and mixed cultures of *E. coli* PHL565 (E.co.), *P. putida* MT2 (P.pu.) and *S. epidermidis* ATCC155 (S.ep.). Cultures were grown overnight at 28 °C in M9sup medium. (A) Direct visualization of biofilm by crystal violet staining. (B) Quantification of biofilm by crystal violet solubilization in ethanol and spectrophotometric determination of Abs at 600 nm. Average of three independent experiments. (C) Quantitative PCR (Q-PCR) analysis of biofilms (resuspended as described in Section 2). White bars: determination of *E. coli* cell concentrations; black bars: determination of *P. putida* cell concentrations. Data are the average of three independent experiments. Presence of a small signal for the *P. putida* primers in pure *E. coli* biofilms would correspond to less than 10 CFU and it is likely to be due to weak cross-reactivity.

initial adhesion of bacterial cells in a porous medium, mimicking bacterial transport and adhesion in an aquifer [17,31]. Consistent with the results of biofilm formation assays on glass, *P. putida* displayed efficient attachment to sand grains, in contrast to poor adhesion shown by *E. coli* and *S. epidermidis*.

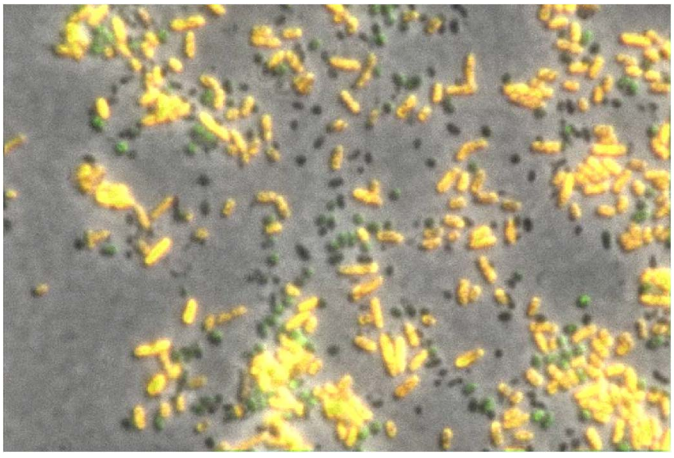

Fig. 3. FISH analysis of cells from *E. coli*/*P. putida* mixed cultures attached to glass slides (magnification = 100×). The slides have been hybridized with an EUB338 bacterial probe FITC labeled (green) and an Enterobacteriaceae specific probe labeled with CY3 (orange-red). *E. coli* cells are stained with both probes and appear bright yellow (lighter rods); *P. putida* cells appear green (darker rods).

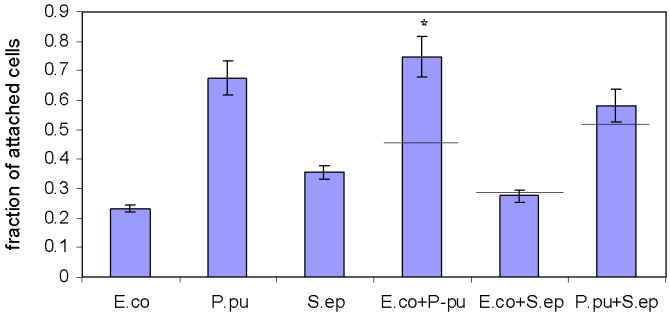


Fig. 4. Initial adhesion to sand columns by either pure or mixed cultures (ratio 1:1) of different bacterial species. The values indicate the fraction of total number of bacteria attaching to sand. For mixed cultures, the bars indicate the expected value in case of lack of interspecies interactions. Values are an average of four different experiments. Differences resulting significant at the Student's test ($t < 0.05$) are marked with a star.

However, *P. putida*/*E. coli* mixed cultures showed synergistic attachment to sand grains, thus suggesting that initial adhesion to surfaces might be enhanced by growth in mixed cultures (Fig. 4).

### 3.3. *Co-adhesion of* E. coli *and environmental bacteria*

The results of biofilm formation experiments with mixed cultures suggest that interaction with *P. putida*, but not with *S. epidermidis*, positively affects surface colonization by the non-adherent *E. coli* PHL565 strain. To investigate whether the positive effect on *E. coli* adhesion is specific to *P. putida* or could also be triggered by environmentally relevant bacterial species, we tested microbial consortia isolated from drinking water catchment areas and distribution systems. The bacterial composition of such consortia was analyzed by fluorescent in situ hybridization (FISH). We could not detect any *E. coli* or *P. putida* or any other important water-borne pathogens such as *Legionella*. Several filamentous bacteria, as well as microorganisms belonging to the $\delta$-Proteobacteria class, could be de-

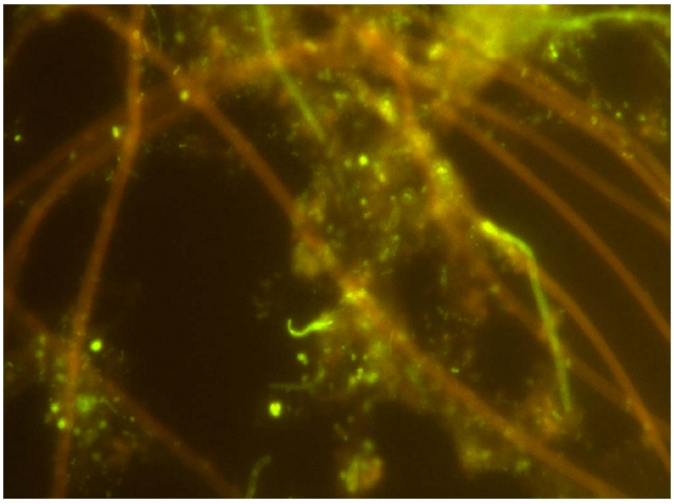

Fig. 5. FISH analysis of microbial consortia from the Spannenburg drinking water catchment area performed using a fluorescein-labeled EUB338 probe able to recognize almost all Eubacteria (magnification = 100×). The sample is constituted by a diverse mixture of long rods and smaller bacteria.

tected in the different consortia. Fig. 5 shows hybridization of the drinking water samples from the Spannenburg site using a probe for Eubacteria and is representative of all the different isolates. All drinking water samples were able to colonize glass tubes and form thin biofilms when grown overnight at 28 °C in M9sup medium, although to a different extent (Figs. 6A, 6B). The sample taken from the drinking water distribution system in Spannenburg was the most proficient in biofilm formation, which included a well-defined ring at the air-medium interface (Fig. 6A, sample 2). Addition of *E. coli* PHL565 to the bacterial communities from drinking water resulted in clear stimulation of glass colonization, similar to what was observed for *P. putida–E. coli* mixed cultures. Quantitative determination of biofilm by crystal violet staining showed an increase in the biomass of the attached cells by about 4-fold (Fig. 6B). Q-PCR determination of *E. coli* PHL565 cells in mixed biofilms from mixed *E. coli*-drinking water bacteria cultures closely resembled the results of *P. putida–E. coli* mixed cultures, showing enrichment in *E. coli* cells ranging from 1.3- (in the Leeuwarden sample) to 2.5-log (in the Spannenburg sample) in the mixed biofilm (Fig. 6C), which corresponds to an increase in *E. coli* cells of between 20- and 300-fold.

## 4. Discussion

In this report, we have shown that adhesion properties vary considerably among *Escherichia coli* strains isolated from the same environment (reservoir waters). Temperature-dependence and strict correlation between adherence and Congo Red binding and *csgB* expression (Table 1) suggest that curli fibers are an important adhesion factor among environmental isolates. This conclusion is also supported by the observation that inactivation of the *csgA* gene, encoding the main curli subunit, leads to an almost complete loss of biofilm formation by the adhesion-proficient WK2 environmental isolate (Fig. 1). Although we cannot rule out the importance of other adhesion factors in environmental strains of *E. coli*, our observations support and complete the results of previous studies showing a strong correlation between the ability to attach to surfaces and curli production in pathogenic strains of *E. coli* [6,23,29,35].

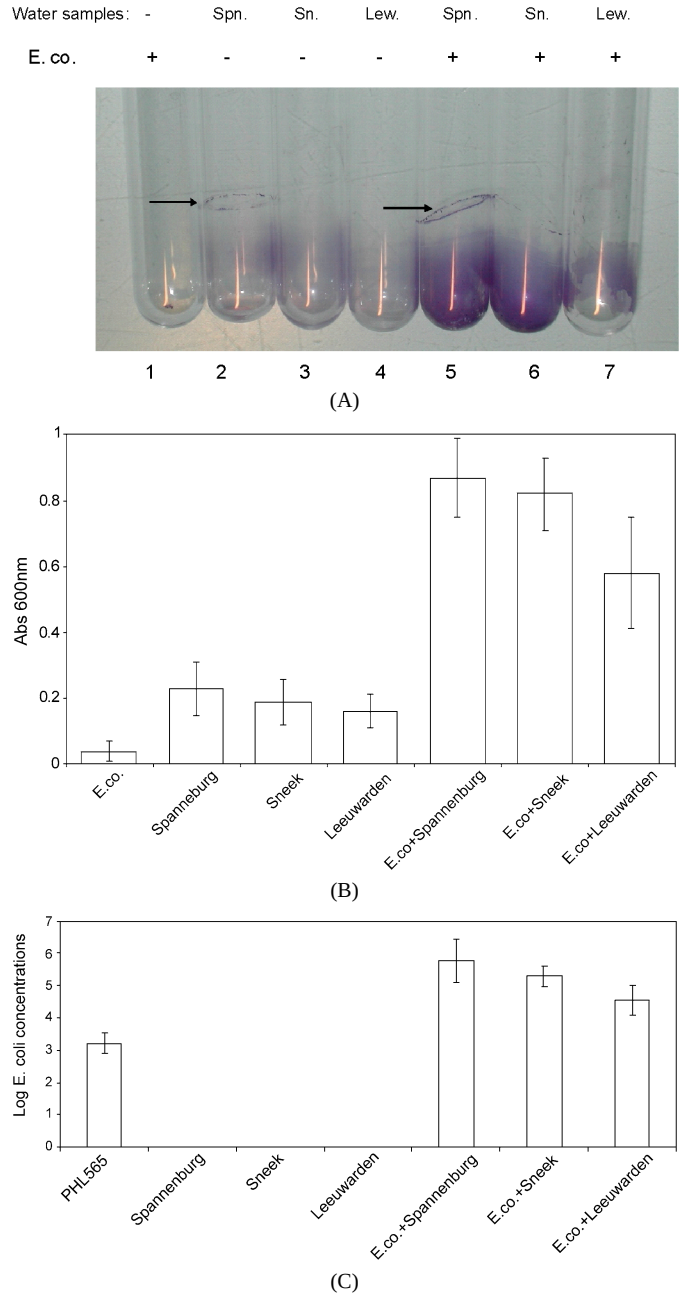


Fig. 6. Biofilm formation on glass tubes by drinking water bacterial consortia in the absence and in the presence of *E. coli* PHL565. Cultures were grown overnight in M9sup at 28 °C. (A) Direct visualization of biofilm by crystal violet staining; the arrows point to the biofilm ring formed at the air-medium interface in the Spannenburg samples. (B) Spectrophotometric quantification of biofilms using crystal violet staining (average of three experiments). (C) Q-PCR quantification of *E. coli* cell concentration (average of three experiments).

Although both the PHL565 laboratory strain and some environmental isolates of *E. coli* showed poor adhesion ability, they might still be able to form mixed biofilms with adhesion-proficient bacteria. Indeed, we could detect the presence of the poorly adhering PHL565 *E. coli* strain in mixed biofilms both

with *P. putida*, an important environmental bacterium, and with bacterial consortia isolated from a drinking water catchment area (Figs. 2, 6). Our results clearly show that PHL565 was not only recruited to a mixed biofilm by adhesion-proficient microorganisms, but even stimulated its formation (Figs. 2, 6). Efficient establishment of the potentially pathogenic bacterium *Listeria monocytogenes* in mixed biofilms with *P. putida* has already been described as increasing its adhesion and persistence [15], thus suggesting that synergistic interactions within mixed biofilms are not unique to *E. coli*. This process appears to be species-specific: indeed, while *E. coli* appears to promote mixed biofilm together with *P. putida*, it fails to do so in *S. epidermidis*/*E. coli* mixed cultures (Fig. 2B). The mechanism of biofilm stimulation by *E. coli* seems to require direct cell–cell contact: addition of *P. putida* spent medium to *E. coli* PHL565 cultures (and vice versa) did not result in increased glass colonization (data not shown), thus suggesting that diffusible signal molecules such as the AI-2 autoinducer, proposed to act in interspecies communication among bacteria [37], are not involved in co-adhesion processes. Results of FISH analysis show that synergistic interaction between *E. coli* and *P. putida* already takes place at an early step of biofilm formation, such as initial adhesion (Fig. 3). It is likely that the initial interaction between *P. putida* and the glass surface allows subsequent recognition of the surface and adhesion by *E. coli*, possibly via production of surfactants or other compounds by the early colonizers. Results of adhesion experiments to sand columns with either pure or mixed *E. coli/P. putida* cultures also support the hypothesis that synergistic interactions favor initial adhesion to solid surfaces (Fig. 4). Sand column tests allow measurement of initial adhesion in an experimental system mimicking transport and adhesion of bacteria in an aquifer, i.e. in conditions similar to the environment [17,31]. We are currently investigating genes and possible extracellular structures in bacterial species that might be involved in enhancement of initial adhesion in mixed cultures.

Results of adhesion experiments in which microbial consortia isolated from drinking water were mixed with *E. coli* PHL565 (Fig. 6) also showed *E. coli*-mediated stimulation of biofilm formation; thus, co-adhesion mechanisms could allow non-adhering strains of *E. coli* to become part of a mixed biofilm in drinking water distribution systems. Identification of environmental microorganisms able to promote co-adhesion with *E. coli* could be extremely valuable; indeed, their monitoring and detection could be an important measure to prevent establishment of *E. coli* and possibly of other pathogenic Enterobacteria in drinking water distribution systems, particularly in settings (i.e., intensive care units and hospital wards) where high standards of microbiologically pure water must be maintained.

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