Association between plasmid carrying an expanded-spectrum cephalosporin resistance and biofilm formation in *Escherichia coli*

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SUMMARY

The formation of biofilm is a universal bacterial survival strategy. Biofilms occur on inert and living support in the natural environment and in industrial installations. This microenvironment leads to the horizontal transfer of genetic material between bacteria by physical contact.
In order to evaluate the relationship between biofilm-forming capabilities, surface characteristics and plasmid content we purified from *Salmonella* a plasmid conferring resistance to cephalosporin and transferred it by electroporation to *E.coli* DH10B originally unable to form biofilm in inert surface. We demonstrated the association between a plasmid conferring resistance to expanded-spectrum cephalosporin and biofilm formation. We also noted that this plasmid influences the cell surface properties and cell motility.

KEY WORDS: Biofilm formation, Plasmid content, Cephalosporin resistance, Cell motility

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INTRODUCTION

The majority of bacteria living in the natural environment are commonly associated with surfaces (Costerton *et al*., 1987). This organization of surface-associated microbial communities forming microcolonies is surrounded by a matrix of exopolymers called biofilm (Costerton *et al*., 1995). Bacteria growing as a biofilm develop significant phenotypical, biochemical and morphological differences from their planktonic counterparts. In particular the most difficult properties of biofilm bacteria are their extreme resistance to treatment with biocides and detergents and their high tolerance of prolonged antibiotic therapy in human infections (Stewart and Costerton, 2001). It is now well documented that biofilms may form on a wide variety of surfaces, including living tissues, in-dwelling medical devices, industrial areas, potable water system piping, or natural aquatic systems (Carpentier and Cerf, 1993).
Bacterial adherence and biofilm formation are strongly influenced by the nutritional environment and are dependent on motility structures such as flagella and pili. These structures contribute to bringing cells together to surfaces. (Wall and Kaisser, 1999, Faille *et al*., 2002). Previous work have shown that exopolysaccharide (EPS) synthesis is induced upon attachment of bacteria to surfaces (Danese *et al*., 2000., Davies and Geesey, 1995, Prigent-Combaret *et al*., 1999). Studies on gram-negative and gram-positive organisms revealed that EPS is required for ini-

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tial attachment to surfaces (McKenny *et al*., 1998, Watnick and Kolter, 1999). Bacteria growing as biofilm produce a repertoire of virulence factors including lipases, proteases, exoploysaccharides, alkaline phosphatases and type IV pili. (Beatson *et al.,* 2002). These factors are also implicated in complex virulence phenotypes such as twitching, swimming and swarming (O'Tool and Kolter, 1998, Deziel *et al.,* 2001).

The identification and characterization of a gene required for biofilm development and motility structures were reported in many organisms (Bechet and Blondeau, 2003, Rashid *et al.,* 2000, Zhiying *et al.,* 2004). It has also been reported that natural conjugative plasmids induce bacterial biofilm development (Ghigo, 2001). The differential expression of a large number of genes is known to occur in the initial steps of biofilm formation. (O'Tool and Kolter, 1998, Pratt and Kolter, 1998). These genes can be carried by plasmids and can exchange with bacteria to modify their behaviour to adapt, survive and initiate a biofilm when environmental conditions change.

Now it is well documented that most pathogenic bacteria in natural, industrial and medical habitats grow as recognized biofilm communities such as *salmonella* spp that are recognized as some of the most common pathogens causing enteritis worldwide which is normally related to unhealthy sanitary conditions (Kingsley and Baumler, 2000).

Many studies have illustrated that the routine use of antibiotics in medical and agricultural circles has resulted in widespread antibiotic resistance and the development of genetic mechanisms efficient for the dissemination of antibiotic gene cassettes, especially within and between species of gram negative organisms (Briggs and Fratamico, 1999).

The present work was undertaken to gather information on plasmid carrying cephalosporin resistance in salmonella and its association with adhesion and biofilm formation. In fact, in industrial processes or in food contact surfaces, salmonella like other bacteria can persist and establish plasmid transfer to sessile bacteria which can cause severe problems for clinicians and hygienists.

This paper reports the phenotypic modification of transformant generated by electroporation of *E.coli* DH10B using a plasmid purified from *Salmonella* isolated from food. We show that in addition to cephalosporin resistance this plasmid expresses factors favouring the access of *E.coli* originally enable to form biofilm in inert surface to establish biofilm communities. The factors implicated in the surface characteristics and cell motility were also investigated.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacteria used in this study were *E.coli* DH10B and *Salmonella spp* resistant to cephalosporin which was isolated from a ground meat.

Tim1 was originally an *E.coli* transformant obtained by electroporation of *E.coli* DH10B by a plasmid conferring resistance to cephalosporin purified from salmonella.

The bacteria were routinely grown aerobically at 37°C on Luria Bertani (L B) medium.

Plasmid Extraction

Plasmid preparation was done following the protocol described by Takahashi and Nagano (1984).

Transformation by electroporation

Approximately 400 ng of plasmid DNA were mixed with 100 µl of electrocompetent cells prepared by the protocol of Sambrook *et al.,* (1989) and the mixture was transferred to a 2 mm gap width electroporation cuvette. After applying a pulse (Settings: 25 µF; 200 Ω; 2,5 kV on a Bio-Rad Gene Pulser™; Bio-Rad), 1 ml of room temperature LB medium was added at once, cells were transferred to a small glass tube and shaken for 2 h at 37°C. The cells were then harvested in a microcentrifuge tube. A total of 900 µl of supernatant was discarded and the cell pellet was resuspended in the residual medium. The entire mixture was then plated on LB + Ceftazidime (20 µg /ml) plate. The plates were incubated for 24 h at 37°C. Controls included cells pulsed without added plasmid DNA.

Biofilm assays

Biofilm assays were carried out using 96-well microtiter dishes using the O'Toole *et al.* (1999) method. Every experiment was performed in triplicate at least three times to confirm the results. Microtiter dishes were covered with a plastic box to prevent evaporation.

Attached cells were visualized after removing the nonadherant bacteria by recurrent washing, staining the wells with 1.0% Cristal Violet for 20 min and rinsing the wells three times with water. Biomass of attached cells (Biofilm) was estimated by solubilisation of the dye in 2 ml of 95% ethanol. The absorbance was measured at 600 nm with a microplate reader PR2100 (Sanofi Diagnostics Pasteur).

Surface characteristics of bacteria cells

Hydrophobic and Lewis acid-base properties of bacteria were assessed by the partitioning method previously described by Bellon-Fontaine *et al*. (1996). This method is based on comparing microbial cell affinity to a polar solvent and microbial cell affinity to nonpolar solvent. The polar solvent can be an electron acceptor or an electron donor, but both solvents must have similar van Der Waals surface tension components. The following pairs of solvents were used: chloroform (electron acceptor solvent), hexadecane (Apolar solvent) and ethyl acetate (electron donor solvent) and decane (Apolar solvent). Due to the surface tension properties of these solvents, differences between the results obtained with chloroform and hexadecane on the one hand, and between ethyl acetate and decane on the other hand would indicate the electron donor and the electron acceptor character of the bacterial surface respectively. The hydrophobic (or hydrophilic) character was estimated by the affinity for apolar solvents.

Experimentally, bacteria were suspended to an optical density of 0.6 at 600 nm (A_0). 2.4 ml of bacterial suspension was vortexed for 30 s with 0.5 ml of the solvent. The mixture was allowed to stand for 30 min to ensure complete separation of the two phases. The optical density at 600 nm of water phase was then measured using a spectrophotometer. The percentage of cells present in each solvent was calculated using the equation: % Affinity = 100 x $(1-A/A_0)$ where A_0 is the optical density at 600 nm of the bacterial suspension before mixing and A is the optical density at 600 nm of the bacteria suspension after mixing.

Motility assays

Swimming: Trypton swim plates (1% trypton, 0.5% NaCl, 0.3% agar) were inoculated with bacteria from an overnight culture in LB agar (1.5% wt/v) plates at 37°C with a sterile toothpick and incubated for 16 to 24 h at 37°C. Motility was then assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

Swarming: media used for assay consisted of 0.5% Difco bacto agar with 8 g/liter Difco nutrient broth, to which 5 g/liter glucose were added. Swarm plates were dried overnight at room temperature. Cells were point inoculated with a sterile toothpick and the plates incubated at 37°C for 24h.

Twitching: cells were stab inoculated with a toothpick through a thin LB agar layer (1% agar) to the bottom of the petri dish. After incubation for 24 to 48 h at 37°C, a hazy zone of growth was observed at the interface between the agar and the polystyrene surface. The ability of bacteria to adhere and form biofilm on the polystyrene surface was then examined by removing the agar, washing unattached cells and staining the attached cells with crystal violet (1% solution).

Statistical analysis

Differences in adherence, biofilm formation and surface properties between wild-type *E.coli* DH10B and electrotransformant strain *Tim1* were analysed by Student's *t* test with Microsoft Excel software.

RESULTS

Isolation of *Tim1* Transformant

Plasmids are among the mobile genetic elements which participate in the dissemination of antibiotic resistance and their transfer leads to a change in the ecological character of bacterial species (Ghigo, 2001).

To assess the relationship between plasmid content of bacteria and biofilm-forming capabilities we generated transformants of *E.coli* DH10B, a classical strain which does not colonize inert surface. After electroporation of *E.coli* DH10B with plasmid associated with resistance to cephalosporin purified from natural *Salmonella* we generated adherent transformant which forms a thick biofilm visible with the naked eye on the walls of glass test tubes (data not shown). Among transformants one clone named Tim1

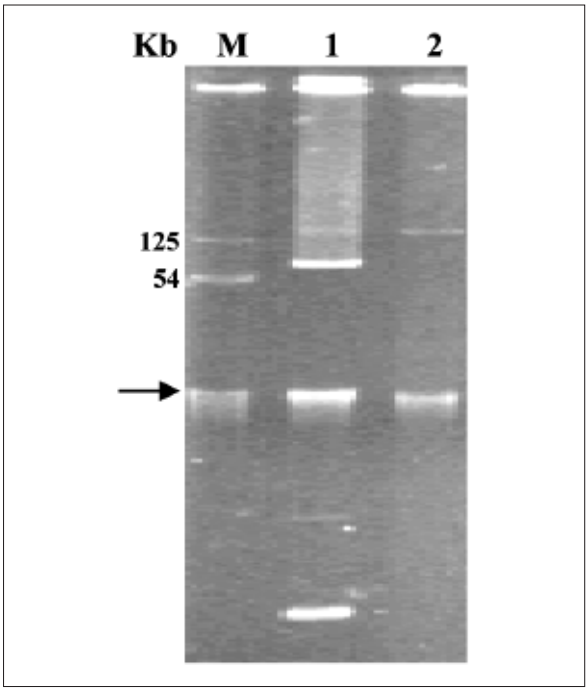

FIGURE 1 - Photograph of 0.8% agarose gel electrophoresis with lanes showing (M) mixture of RP4 (54 kb) and pIP 173 (125 kb) plasmids as reference molecular mass markers; (1) plasmid content of *Salmonella* and (2): plasmid content of *Tim1*, transformant generated by electroporation of *E. Coli* DH10B with plasmid purified from *Salmonella*. The arrow indicates the chromosomal DNA.

resistant to cephalosporins exhibits a strong and rapid adherence on glass.

Figure 1 shows a plasmid profile of *Salmonella* and a transformant Tim1. Plasmid bearing adherence character has a high molecular weight (Superior to 125 Kb). Previous works have reported that plasmids of high molecular weight were implicated in resistance to cephalosporins (Carattoli *et al.*, 2002, Rankin *et al.,* 2002).

Biofilm formation by *Tim1*

Escherichia coli DH10B and its transformant *Tim1* were grown in LB broth in polystyrene wells. After 6 h of growth in static conditions the planktonic cells were removed by vigorous rinsing with water and the extent of biofilm formation of strains was analyzed macroscopically by staining with crystal violet. Figure 2 showed that the wild type strain *E.coli* DH10B did not form a detectable biofilm comparatively to its transformant *Tim1* which forms the best biofilm on

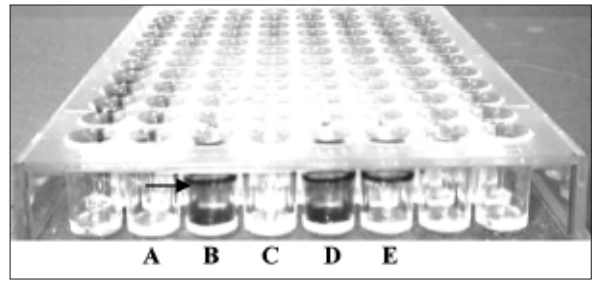

FIGURE 2 - Biofilm formation in the wells of a polystyrene microtiter plate. The wells contained *E. coli* AL52 (A) a negative control, *Salmonella* (B), *E. coli* DH10B (C), *Tim1* (D) and Pseudomonas aeruginosa ATCC 27853 (E) a positive control. After overnight incubation at 30°C, the wells were rinsed out and stained with crystal violet. The biofilm is concentrated at the interface between the air and the liquid medium (arrow).

polystyrene in LB broth. This observation indicates that natural plasmid expressed factors that induced planktonic *E.coli* to form a biofilm. This result is in agreement with Ghigo who reported that a conjugative plasmid of natural population of gram negative bacteria are implicated in the biofilm formation process.

Kinetics of biofilm formation

The dynamics of biofilm formation was measured for the three strains by culturing them in nonagitated microtiter dishes. As shown in figure 3, the wild type strain did not form a significant biofilm even after 8 h of incubation. In contrast the *Tim1* transformant quickly adhered and formed a dense biofilm within a few hours. The biofilm formed by *Tim1* then rapidly dispersed, probably following exhaustion of the growth substrate.

Hydrophobicity and Lewis acid-base properties of cell surface

Hydrophobicity: the two nonpolar solvents hexadecane and decane were used to estimate the cell surface hydrophobicity of *Salmonella, E. coli* DH10B and *Tim1* transformant. Bacterial affinity to the two nonpolar solvents is presented in table 1.

The affinity of *Salmonella* and transformant strains was high to the two nonpolar solvents. These results demonstrated that both strains exhibited a relative hydrophobic property. In contrast the affinity of *E. coli* DH10B to the two solvents was very low. This result shows that this strain is very hydrophilic.

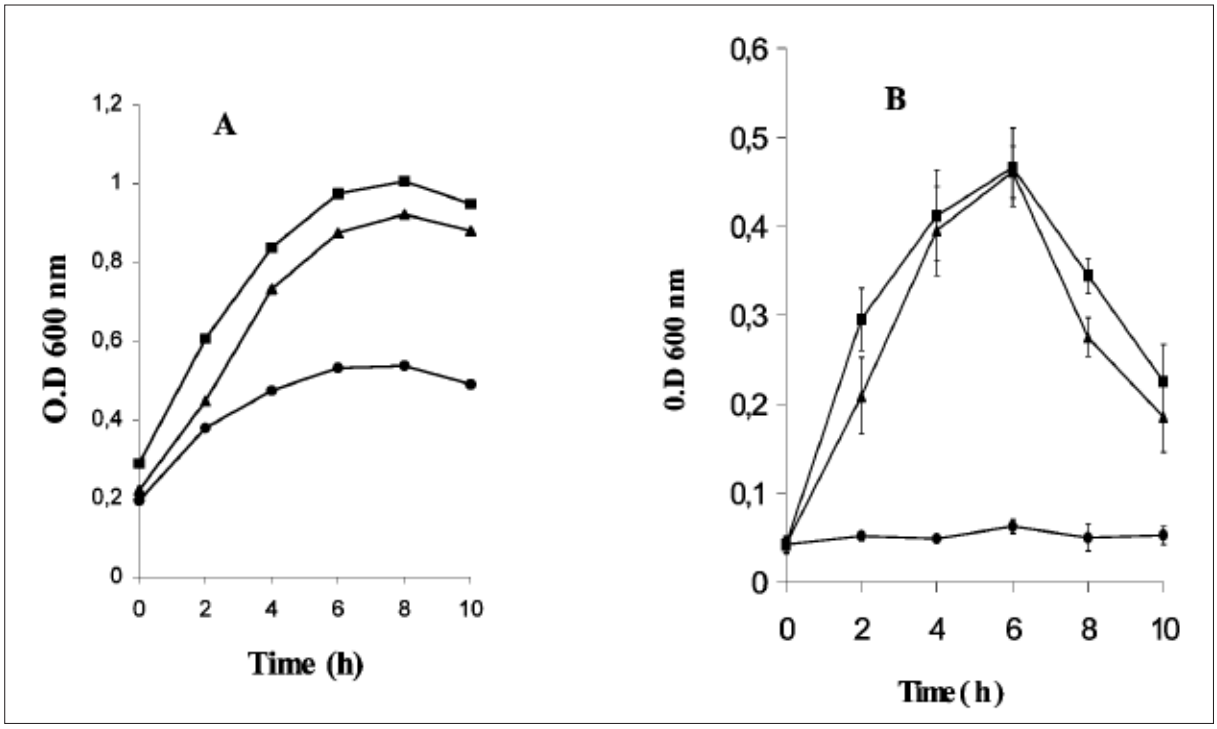

FIGURE 3 - Kinetics of biofilm formation. *E. coli* DH10B (•), *Salmonella* (▲) and *Tim1* (■). Strains were cultivated in polystyrene plates at 37°C without agitation. At the indicated time intervals samples were harvested to determine growth (A) and biofilm formation after crystal violet staining (B) as described in material and methods. (Error bars show standard deviations).

Lewis acid–base properties: the four solvents: chloroform, hexadecane, ethyl acetate and decane were used to estimate the Lewis acid–base properties (i.e. electron donor/electron acceptor character). The results are presented in table 2. The affinity of all bacteria was higher with chloroform (an electron acceptor solvent) than with hexadecane (nonpolar solvent) indicating the electron donor character of these bacteria.
According to table 2, the cells of *E. coli* DH10B adhered more to the ethyl acetate (an electron donor) than to decane (nonpolar solvent) showing that this strain exhibits an electron acceptor character (acid). In contrast, the affinity of *Tim1* transformant and *Salmonella* was lower with ethyl acetate than with decane indicating that no electron acceptor was exhibited by these strains.
The results obtained in this study (Tables 1 and 2) show that the cell surface hydrophobicity and the Lewis acid-base properties of *E. coli* DH10B were modified when the plasmid of *Salmonella* was transferred to this strain. This result shows that the *E. coli* DH10B became hydrophobic and the electron donor/ electron acceptor decreased

TABLE 1 - The cell surface hydrophobicity of *Salmonella*, *E. coli* DH10B and the Transformant *Tim1* (Average ± Standard deviation n=3)

Strains	*% affinity to hexadecane*	*% affinity to decane*
Salmonella	41.6 (± 1.9)	59.4 (± 1.5)
E. coli DH10B	3.6 (± 0.4)	4.3 (± 0.7)
Transformant (*Tim1*)	62.1 (± 1.9)	81.9 (± 2.9)

TABLE 2 - Lewis acid-base properties of the cell surface (Average ± Standard deviation n=3).

Strains	*% Affinity with chloroform*	*% Affinity with hexadecane*	*% Affinity with ethyl acetate*	*% Affinity with decane*	*Electron donor*	*Electron acceptor*
Salmonella	75.4 (± 5.8)	41.6 (± 1.9)	38.2 (± 2.1)	59.4 (± 1.5)	34.0	0
E.coli DH10B	80.1 (± 2.1)	3.6 (± 0.4)	27.4 (± 3.0)	4.3 (± 0.7)	76.5	23.1
Transformant (Tim1)	85.0 (± 1.8)	62.1 (± 1.9)	76.7 (± 3.2)	81.9 (± 2.9)	22.9	0

when the plasmid of *Salmonella* was transferred to this strain.

Cell motilities

A comparative study of the behaviour of the three strains was done. We obtained that swarming motilities (generated by lateral flagella) characterized by a migration of the bacteria away from the point of inoculation on agar plates were observed for the *Tim1* transformant and *Salmonella*, but the *E.coli* DH10B remained near the point of inoculation and did not show a swarming motility (Figure 4.1). Swimming motility due to the presence of polar flagella was also detected for *Tim1* transformant and *Salmonella* on a swimming plate but this phenotype was not observed in *E.coli* DH10B (Figure: 4.2). Another phenomenon, twitching correlated with the presence of functional polar pili was investigated for the three strains. The results did not show a difference between *Salmonella*, *E.coli* DH10B and its transformant. In fact, the zone of spreading growth between the agar and the bottom of the petri dish which is typical of twitching motility was very limited and less prominent than the spreading zone occurring at the agar surface, even after prolonged incubation (Figure 4.3).

DISCUSSION

Biofilm is the prevailing microbial lifestyle of most bacteria, and is ideally suited to exchange by conjugation of genetic material of various origins. Generally the mechanisms involved in the resistance of biofilm bacteria to antimicrobials are complex and still not fully understood, but the problem increases when bacteria have a plasmid carrying direct resistance to antibiotics and able to form biofilms. The aim of this work was to elucidate the relationship between plasmids bearing cephalosporin resistance and biofilm formation.

The rate of conjugation in biofilm is 1000 fold higher than that determined by classical plating

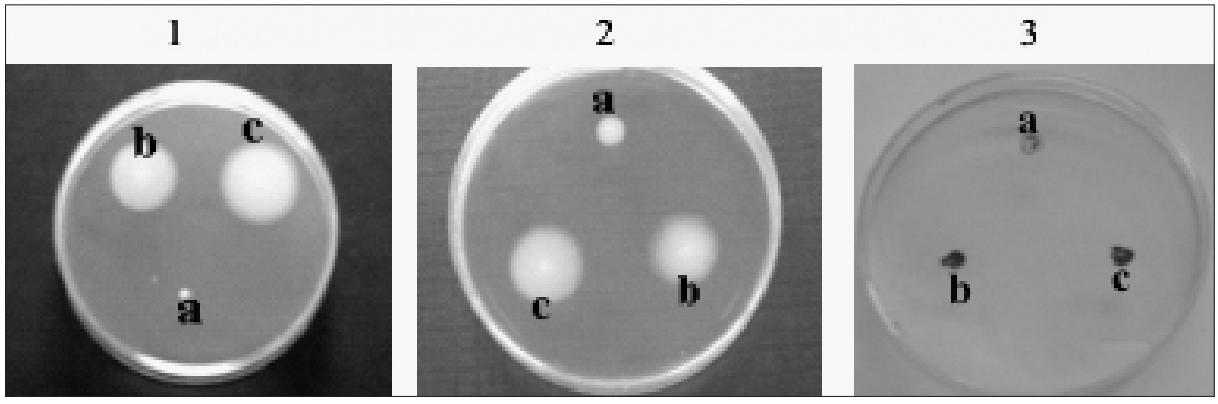

FIGURE 4 - Comparison of motility phenotypes of *E. coli* DH10B (a), *Tim1* (b) and *Salmonella* (c) **1**: Swarming motility on a 0.5% agar plate; **2**: Swimming motility on a tryptone swim plate (0.3% agar); **3**: Twitching motility observed after crystal violet staining of cells that remained attached to the polystyrene surface after removing the agar layer and washing with water.

techniques (Hausner and Wuertz, 1999). Previous works have reported a relationship between biofilm forming capabilities and antibiotic resistant phenotypes. Delissalde and Amabile-Cuevas, (2004) found that strains of *Pseudomonas aeruginosa* which produce biofilms are slightly more resistant to antibiotics. On the other hand, no relationship was observed between plasmid resistance and biofilm formation. Our work shows the direct implication of plasmid conferring resistance to expanded-spectrum cephalosporin in bacterial attachment and biofilm formation. We also investigated the relationship between this plasmid and the cell surface structures by studying the cell motilities and physicochemical properties.

After electroporation experiments, we observed that the *Tim1* transformant exhibits biofilm formation properties in addition to expanded spectrum cephalosporin resistance. This result suggests that in addition to antibiotic resistance, this plasmid also contains gene(s) involved in biofilm formation.

It has been already reported that resistance to cephalosporin in enterobacteria was encoded on large plasmids with a molecular weight ranging from 20 to 160 kb (Carattoli *et al*., 2002, Rankin *et al.,* 2002). It was also demonstrated that plasmids from different incompatibility groups led to a biofilm and that the induction of biofilm formation is not restricted to a specific incompatibility group nor to a specific type of pili (Ghigo, 2001). Our study also reports that a plasmid larger than 125 kb confers resistance to cephalosporins and bears biofilm properties.

Concerning the surface properties of *Salmonella, E. coli* DH10B and transformant *Tim1*, the results show that *E. coli* DH10B carried the hydrophobicity and the electron donor/ electron acceptor character of *Salmonella* when the plasmid of this strain was transferred to *E. coli* DH10B. The transfer of the *Salmonella* plasmid to *E. coli* DH10B could indicate that these strains could be closely genetically related. Giovannacci *et al.* (2000) demonstrated that three strains of genetically related *Listeria monocytogenes* exhibited similar surface properties. (Reid *et al.* 1999) also demonstrated a correlation between the genetic and physico-chemical surface characteristics of strains of *Lactobacillus rhamnosus*. So, the similar surface properties observed for *Salmonella* and the transformant *Tim1* could be explained by the fact that these strains are genetically related. Many works showed that strains containing plasmids adhered better than similar strains without plasmids. (Reid *et al*., 1999, Tewari *et al.*, 1985). But so far no work has confirmed the specific correlation between plasmid content and physicochemical surface characteristics. A previous study of a collection of *Staphylococci* identified two pairs which had pair-wise different plasmid profiles (Busscher *et al*., 1994). In that study, a hierarchical cluster analysis based upon squared euclidian distances revealed that a 56 kb plasmid might be involved in the modulation of cell surface hydrophobicity. In our work we report the transfer of the plasmid to a strain which is originally unable to adhere to inert surface and thereby confirm the specific correlation between plasmid content and cell surface properties.

Our results suggest that our plasmid may be involved in extracellular structures which have been described in many bacteria as essential for interaction with inert surfaces and biofilm formation such as type IV pili in *Pseudomonas aeruginosa* (O'Tool and Kolter, 1998), thin aggregative fimbriae (SEF 17) in *Salmonella* (Austin *et al.,* 1998), type 1 pili and curli in *E.Coli* (Pratt and Kolter, 1998, Prigent *et al*., 2000), SSP adhesin in *Staphylococcus epidermis* (Hellman *et al.,* 1997, Timmerman *et al*.,1991) and type 3 pili in *Klebsiella pneumoniae* (Di Martino *et al.,* 2003*)*.

Our results also indicate that the plasmid studied contributes to the synthesis of flagella. In fact, it has been reported in many work that two different types of motility are mediated by flagella. In addition to swimming motility in liquid, flagella mediated swarming motility on solid surfaces or in viscous conditions. Several studies have reported that those types of motility contribute to bacterial colonization of host cell surfaces or abiotic surfaces, and play a significant role in biofilm formation (Kirov, 2003). Hence it is reasonable to think that biofilm formation is a consequence of bacterial motility: surface colonization is initiated by the attachment of individual cells to a surface (swimming by polar flagella) followed by their surface migration (twitching and/or swarming), replication to form microcolonies and quorum sensing regulated differentiation into the mature biofilm.

O'Toole and Kolter (1989) confirmed the role of these flagella by reporting that the non biofilm forming mutant isolated from *Pseudomonas aeruginosa* and *E.coli* were defective in flagellar-mediated motility.

Several studies in our laboratory are now in progress cloning and sequencing to characterize the region of our plasmid supplying aggregative properties that stimulate biofilm formation. The spread of strains with such plasmid may have serious clinical consequences, since expanded-spectrum cephalosporins are the antibiotics of choice for invasive *Salmonella* infections in children. In addition, plasmid transfer shows increased efficiency in the biofilm population relative to planktonic population. This may contribute to increased contamination of different origins in several sectors since bacteria in biofilm are more resistant to disinfectant than bacteria living in the planktonic population.

In conclusion, we think that the identified plasmid deserves extensive study such as a subcloning of the gene implicated in adhesion and establishment of the relationship between this gene and multiple fimbrial adhesins especially as many works report a close relationship between virulence and adhesion of *Salmonella* to intestinal mucosa.

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