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Biofilm Formation as a Virulence Determinant of Uropathogenic *Escherichia coli* Dr$^+$ Strains

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Abstract

Urinary tract infections are the most common health problem affecting millions of people each year. Uropathogenic *Escherichia coli* (UPEC) strains are the major factor causing lower and upper urinary tract infections. UPEC produce several virulence factors among which are surface exposed adhesive organelles (pili/fimbriae) responsible for colonization, invasion and amplification within uroepithelial cells. The virulence of the uropathogenic *E. coli* Dr$^+$ IH11128 is associated with Dr fimbriae belonging to the Dr family of adhesins (associated with diarrhea and urinary tract infections) and a DraD protein capping the linear fiber at the bacterial cell surface. In this study we revealed that biofilm development can be another urovirulence determinant allowing pathogenic *E. coli* Dr$^+$ to survive within the urinary tract. *E. coli* strains were grown in rich or minimal media, allowed to adhere to abiotic surfaces and analyzed microscopically by staining of cells with cristal violet. We found that both Dr fimbriae and DraD, exposed at the cell surface in two forms, fimbria-associated or fimbria non-associated, (DraE$^+$/DraD$^+$, DraE$^+$/DraD$^-$ or DraE$^-$/DraD$^+$ *E. coli* strains) are required for biofilm formation. Additionally, we demonstrated the biofilm formation capacity of *E. coli* strains deficient in the surface secretion or production of the DraE adhesin.

Key words: *E. coli* Dr$^+$, aggregation, biofilm, *dra* gene cluster, uropathogenic

Introduction

Many bacterial strains live in sessile communities called biofilms. Biofilms are compact microbial communities consisting of organisms adherent to each other and a target surface (Geesey *et al*., 1977; Costerton *et al*., 1994; 1995). Biofilms are formed by a number of punctuated microcolonies separated from each other by liquid channels responsible for supplying nutrients (influx) and removal of the metabolic products (efflux). Biofilm development occurs in a few steps. First, the planktonic bacteria sessile on a biotic/abiotic surface adhere and create a cluster. Then the cells form antimicrobial resistant colonies. Some cells probably leave the biofilm colonies to settle on a surface and create new agglomerations (Costerton *et al*., 1994; 1995).

In fact, bacterial biofilms can establish on any solid surface of inorganic or organic nature spanning a wide spectrum of environments (Schembri *et al*., 2001; Schembri and Klemm, 2001). Autoaggregation of bacterial cells can create more beneficial conditions for colonization (Soto *et al*., 2007; Naves *et al*., 2008). Diseases connected with the ability of bacteria to form biofilms are generally chronic and difficult to treat because of biofilm resistance to antimicrobial agents compared to their planktonic (free-living) counterparts. The greater resistance to antibiotic therapy than the planktonic colonies is probably the effect of lack of penetration of biofilms by antibiotics (Costerton *et al*., 1999). *Escherichia coli* strains causing prostatitis produce biofilm communities *in vitro* more frequently than uropathogenic strains involved in cystitis and pyelonephritis (Soto *et al*., 2007). In those infections type 1 pili are considered to be an important factor in the first step of biofilm formation. Type 1 pili mediate binding to the host receptor and invasion of bacteria into bladder epithelial cells which activate a gene cascade essential for the formation of intracellular bacterial communities (Anderson *et al*., 2003; Justice *et al*., 2004).

Urinary tract infections (UTI) are among the most common bacterial infections in humans and affect millions of people each year. The most frequent etiologic

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agent of urogenital infections is uropathogenic *E. coli*, UPEC accounting for 65 to 90% of cases (Millar and Cox, 1997). Urovirulence factors associated with UPEC include surface (type 1 and P pili, S and F1C fimbriae, Dr family of adhesins) and exported virulence determinants (toxins, siderophores). Tissue invasion and biofilm formation can be other urovirulence factors which allow the bacterial cells to survive and persist a long time within the upper or lower urinary tract (Oelschlaeger *et al*., 2002; Emödy *et al*., 2003; Arisoy *et al*., 2006; Yamamoto, 2007).

E. coli strains bearing the Dr family of adhesins account for 40% of pylenophritis cases in the third trimester of pregnancy, 50% of chronic diarrhea cases in children and 20% of recurrent urinary tract infections in young women (Foxman *et al*., 1995; Goluszko *et al*., 1997). The Dr family includes fimbrial (DraE adhesin) and afimbrial adhesins (AFA – I, –II, –III and –IV) (Servin, 2005; van Loy *et al*., 2002). The *dra* gene clusters share a highly conserved region, including the *draF*, *draA*, *draB*, *draC*, and *draD* genes (Nowicki *et al*., 1989). The *draE* gene, highly heterogeneous within the Dr family of adhesins, is the adhesin-encoding gene (Zalewska-Piątek *et al.*, 2008). Dr haemaglutinin is the only member of the Dr family of adhesins that has the unique ability to bind to DAF and type IV collagen receptors. The DraD protein binds to β_1 integrins, a common receptor for AfaD invasins (Garcia *et al*., 1996; Plancon *et al*., 2003; Zalewska-Piątek *et al*., 2008). The interactions with the host receptors are essential for the subsequent internalization of bacterial cells and the maintenance of chronic infections (Plancon *et al*., 2003; Cota *et al*., 2006). The adherence capacity of *E. coli* Dr^+ can also stimulate bacterial aggregation, which can be the first step of biofilm development and formation of cell microcolonies (Zalewska-Piątek *et al*., 2008).

In this paper, we present the role of Dr fimbriae and DraD protein as virulence factors exposed at the surface of uropathogenic *E. coli* Dr^+ strains, in biofilm formation under different nutrient conditions. The investigations involved the use of *E. coli* strains (of laboratory and clinical origin) expressing Dr fimbriae with or without DraD as a capping fimbrial subunit ($DraE^+$ and $DraD^+/DraE^+$ and $DraD^-$, respectively). Because the DraD protein can be expressed in two forms, fimbria-associated and fimbria non-associated, as a protein adhesive sheath surrounding the bacterial cells, it was very important to reveal the predisposition of UPEC to form structured bacterial communities *in vitro* without the fimbrial urovirulence determinant ($DraE^-$ and $DraD^+$). The ability of the selected *E. coli* strains to create aggregates of bacterial cells was confirmed by analysis of biofilm growth in rich or minimal media and light microscopy of cells stained with cristal violet (CV).

Experimental

Materials and Methods

Bacterial strains, plasmids and reagents. The expression of genes encoded by a *dra* gene cluster was carried out in laboratory *Escherichia coli* BL21(DE3) (Novagen, Nottingham, UK), or clinical *E. coli* IH11128 (Goluszko *et al*., 1997), DR14 and DR14/*gspD* strains (Zalewska-Piątek *et al*., 2008).

E. coli BL21(DE3) is a λDE3 strain, which carries the gene for T7 polymerase under *lacUV5* promoter control. *E. coli* IH11128 is a strain of clinical origin (isolated from human with pylenophritis) bearing Dr fimbriae (Goluszko *et al*., 1997). *E. coli* DR14 is an insertional *draC* mutant strain, described previously (Goluszko *et al*., 1997). The *E. coli* DR14/*gspD* strain is a *gspD* mutant (with a *gspD* gene knockout) of *E. coli* DR14, described previously (Zalewska-Piątek *et al*., 2008).

The laboratory *E. coli* strains harboring the *dra* gene cluster and its mutants with an inactivation in the *draE*, *draD* or *draC* genes grew in either LB or M63 minimal medium supplemented with 0.2% glucose and 1% LB. The clinical *E. coli* strains were cultivated only in M63 minimal glucose medium.

Plasmids pCC90 carrying the *dra* gene cluster with its promoter region and regulatory genes (*draF-draA*) upstream of a *draB* gene deleted, and pCC90D54stop (the DraE-negative mutant), with a mutated *draE* gene were provided by S. Moseley, University of Washington, Seattle, WA, USA (Carnoy and Moseley, 1997). Plasmids pCC90DraDmut (the DraD-negative mutant), with a mutated *draD* gene, and pCC90DraCmut (the DraC-negative mutant), with a mutated *draC* gene were described previously (Zalewska *et al.*, 2005).

PVC (polyvinyl chloride) dishes (96-well; Falcon 3911 microtest III flexible assay plates) and glass coverslips were obtained from Becton Dickinson (Franklin Lakes, NJ). 1% crystal violet (CV), a dye which stains attached cells but not PVC was purchased from Merck (Darmstadt, Germany).

Growth of biofilm in rich and minimal media. Ten ml cultures of the *E. coli* strains of interest were grown in LB at 37C° without shaking for 24 h. Bacterial strains were then subcultured (1:100) into 200 µl of fresh LB and minimal medium M63 supplemented with 0.2% glucose and 1% LB (M63 minimal glucose) in 96-well plates. The cells were grown for an additional 24 h at 37°C without shaking. After that time planktonic cells were removed by three-time rinsing with PBS (phosphate-buffered saline) and then the biofilm was stained with 200 µl of 1% (w/v) CV. After washing, the A_{531} was determined. Strains presenting a blank corrected mean absorbance value of >0.1 were considered as positive.

Light microscopy of biofilm. Strains were grown in LB for 24 h at 37°C without shaking. Then the bacterial cultures were subcultured into LB or M63 minimal glucose media. The biofilm assays were performed by using either polystyrene 6-well culture dishes with glass coverslips. Two ml of LB or M63 minimal glucose media were add to each well simultaneously with 40 µl of the bacterial overnight culture. The culture dishes were incubated overnight at 37C° without shaking. After 24 h the wells were washed 3 times with PBS. Biofilm formation was visualized by first fixing the bacteria with 10% (v/v) formalin for 10 min and then staining with 300 µl of 1% CV for 5 min, washing with PBS and air-drying. The cover slides were removed from the wells, mounted on microscopic slides and observed by a light microscopy using a 40 × objective lens and a Olympus BX-60 microscope.

Results

Biofilm formation under various growth conditions (rich and minimal environments). The ability of uropathogenic *E. coli* Dr$^+$ and Dr$^-$ strains to form biofilms was determined through the growth of bacteria in either LB or M63 minimal glucose media on PVC plates. Bacterial cells attached to the abiotic surfaces were visualized after staining with CV (observation of stained purple cells) (Fig. 1, part A and B). In our investigations we used 8 different *E. coli* strains of laboratory and clinical origin. The laboratory *E. coli* BL21(DE3) strain (Fig. 1a) and the same strain transformed with pCC90 encoding the *dra* cluster without a regulatory region (the DraE and DraD positive strain) (Fig. 1b) were used as a negative and positive control of biofilm formation, respectively. The *E. coli* BL21(DE3)-pCC90DraDmut containing the *dra* gene cluster with an inactivated *draD* gene (the DraD negative strain) (Fig. 1c) was employed to reveal the role of fimbrial polymer composed of only the DraE adhesin, without the DraD capping subunit (DraE$^+$ and DraD$^-$), in formation of bacterial communities. The *E. coli* BL21(DE3)-pCC90DraCmut and *E. coli* BL21(DE3)-pCC90D54stop harboring the *dra* operon with an inactivated *draC* or *draE* gene, respectively (the DraC and DraE negative strains) (Fig. 1d-e) were used to determine whether the DraD protein non-associated with Dr fimbriae might also be important for the adherence to abiotic surfaces under static growth conditions. The results obtained were compared with the clinical *E. coli* IH1128 bearing Dr fimbriae (Fig. 1g), its insertional *draC* mutant DR14 (DraC serves as a polymerization platform of Dr fimbriae) (Fig. 1h) and D14/*gspD* with the *gspD* gene disruption (GspD is responsible for the DraD secretion across the outer membrane on the bacterial cell surface) (Fig. 1f).

In case of the above laboratory *E. coli* strains we could observe biofilm formation in both LB (Fig. 1b-e, part A) and glucose-minimal medium (Fig. 1b-e, part B). On the contrary the clinical strains revealed the effect of bacterial aggregation only in a glucose-minimal medium (Fig. 1g-h) because of the methylation-dependent phase-variation mechanism which controls the expression of the genes encoding urovirulence determinants and allowing the alternation between Dr fimbriae plus (phase ON), and Dr fimbriae minus (phase OFF) states. The biofilm phenotypes were exhibited by *E. coli* strains harboring the whole *dra* gene cluster and also its mutants with an inactivation in the *draE* or *draC* genes (expressing only DraD protein at the bacterial cell surface) or the *draD* gene (with surface exposition of Dr fimbriae). Additionally, the moderately visible differences in biofilm formation was shown in case of bacterial strains expressing Dr fimbriae with (Fig. 1b) or without the

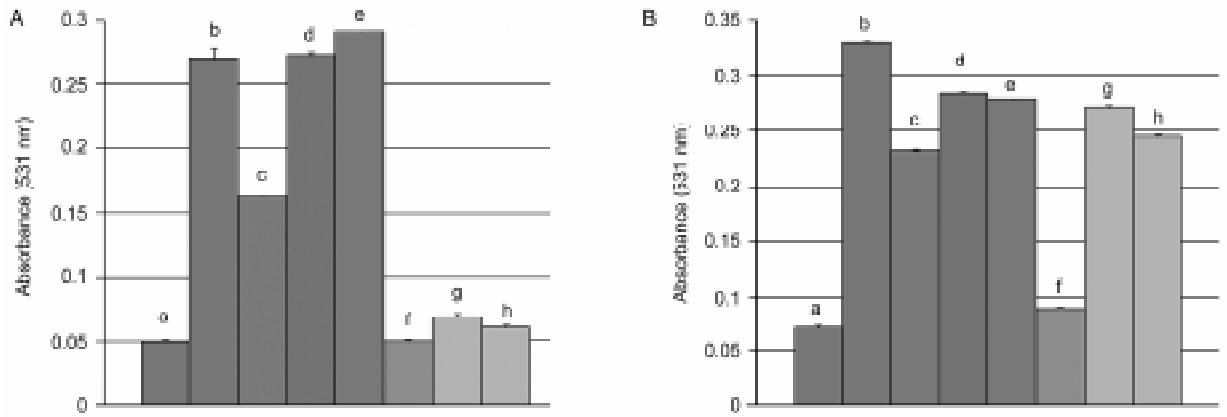

Fig. 1. Analysis of growth of biofilm in rich LB (A) and glucose-minimal medium (B).

E. coli biofilms were stained with CV and the A_{531} of each CV sample was determined. Each bar represents the mean ± SEM from three independent experiments: a) *E. coli* BL21(DE3); b) *E. coli* BL21(DE3)-pCC90; c) *E. coli* BL21(DE3)-pCC90DraDmut; d) *E. coli* BL21(DE3)-pCC90DraCmut; e) *E. coli* BL21(DE3)-pCC90D54stop; f) *E. coli* DR14/*gspD*; g) *E. coli* IH11128; h) *E. coli* DR14.

DraD as a capping fimbrial domain (Fig. 1c) in comparison with the strains exposing only DraD ($DraE^+$/$DraD^+$, $DraE^+$/$DraD^-$ or $DraE^-$/$DraD^+$ *E. coli* strains, respectively) (Fig. 1d-e, h) on the cell surfaces.

Microscopic analysis of biofilm formation. Both laboratory and clinical *E. coli* strains were analyzed by the light microscopy to confirm the role of Dr fimbriae and DraD protein during biofilm formation. The strains were grown for 24 h in LB or glucose-minimal media (laboratory and clinical strains, respectively) on glass coverslips, stained with CV and observed under the light microscopy. The bacteria forming biofilm were visible as cell clusters (Fig. 2). The effect of bacterial aggregation was not exhibited by the *E. coli* BL21(DE3) (Fig. 2A) and *E. coli* DR14/*gspD* with the *draC* and *gspD* gene knockout (Fig. 2F). The *E. coli* BL21(DE3)-pCC90 and the wild-type *E. coli* IH11128 strains (Fig. 2B and G) were observed as the dark clusters of cells. The same effect was displayed by the *E. coli* BL21(DE3)-pCC90DraCmut and *E. coli* DR14 (the DraC negative strains) (Fig. 2D and H), *E. coli* BL21(DE3)-pCC90D54stop (the DraE negative strain) (Fig. 2E) or *E. coli* BL21(DE3)-pCC90DraDmut (the DraD negative strain) (Fig. 2C). Additionally, in case of $DraE^-$ and $DraD^+$ (Fig. 2D-E and H) or $DraE^+$ and $DraD^-$ *E. coli* strains (Fig. 2C), the clusters of cells could be visualized as interspersed layers of various density. The microscopic studies revealed that Dr fimbriae are critical for the interaction of *E. coli* with abiotic surfaces. We also showed the instrumental role of the DraD protein, located alone at the bacterial cell surface or associated with the fimbrial organelles, in the development of biofilm.

Discussion

UTI are the most common urologic diseases in humans. Uropathogenic *E. coli* strains are the most frequent etiologic agent of UTI. The strains exhibit various virulence determinants required for the process of colonization of lower or upper urinary tract and persistence of the bacterial infections. Among these urovirulence factors are adhesive subunits, the structural elements, of homo- or heteropolymeric structures exposed at the surface of bacterial cells (Foxman *et al*., 1995; Arisoy *et al*., 2006; Yamamoto *et al*., 2007). The adhesive structures can be also associated with tissue invasion and biofilm development during the course of UTI. Biofilm formation is classified as a pathogenic determinant of uropathogenic *E. coli* strains which allows bacteria to adhere to any abiotic or biological surfaces (Costerton *et al*., 1995; Soto *et al*., 2007).

One of the *E. coli* urovirulence factors is the Dr family of adhesins responsible for lower and upper UTI, especially in young women and children. This adhesive family includes DraE, DaaE, AfaE-I, AfaE-III, AfaE-V, NfaE and others (Servin, 2005). DraE adhesin, a subject of our studies, is encoded by the *dra* gene cluster which also contains *draF*, *draA*, *draB*, *draC*, *draD* and *draP* genes (Nowicki *et al*., 1989). In clinical *E. coli* Dr^+ strains DraE forms linear polymers capped at the tip by the DraD protein, enabling the bacteria to enter and replicate within epithelial cells (Anderson *et al*., 2004; Piątek *et al*., 2005; Zalewska *et al*., 2005). The internalization of *E. coli* by eukariotic cells *via* a zipper-like mechanism is dependent on dynamic microtubules, lipid rafts, and $\alpha_5\beta_1$ integrin. The interactions with the host receptors (the initial step in colonization) and invasion are required for recurrent and chronic UTI (Goluszko *et al*., 1997; Guignot *et al*., 2001; Kansau *et al*., 2004).

In this study, we demonstrated for the first time the biofilm development by uropathogenic *E. coli* strains harboring the *dra* gene cluster. The formation of bacterial communities by laboratory and clinical *E. coli* strains was analyzed under rich and minimal growth conditions. It was connected with the expression of virulence determinants often regulated by the various environmental stimuli. Some of the stimuli can suppress the transcription of the virulence genes such as fimbrial genes (Mekalanos, 1992; White-Zigler *et al*., 2000).

In case of clinical *E. coli* IH11128 and DR14 isolates, containing the whole *dra* gene cluster with a regulatory region including the *draF-draA* genes, biofilm formation was not observed in LB medium. The results obtained were in accordance with earlier published investigations which revealed that rich medium, LB, repressed transcription of *pap*, *daa* and *fan* gene clusters encoding type P pili, F1845 fimbriae and K99 fimbriae, respectively (White-Zigler *et al*., 2000). The analyzed operons were also under the control of phase-variation mechanism, which allows the bacteria a transition between two expression states: phase ON characterized by expression of pili/fimbriae and phase OFF blocking the expression of pili/fimbriae (Bilge *et al*., 1993; van der Woude *et al*., 1992). The mentioned mechanism is controlled at the transcription level and is associated with the formation of specific DNA methylation patterns of GATC boxes. The GATC I site is non-methylated in phase ON whereas the GATC II box is non-methylated in phase OFF (van der Woude *et al*., 1992; van der Woude and Low, 1992). The exactly role of repression effect of LB medium is not determined. However, it is assumed that the bacterial growth in this medium increases the rate at which the cells expressing fimbriae alternate to phase OFF (White-Ziegler *et al*., 2000). The *dra* gene cluster of *E. coli* Dr^+ also contains two GATC boxes (spaced by 102 bp) between *draF* and *draA* genes which are conserved among pili/fimbrial operons of

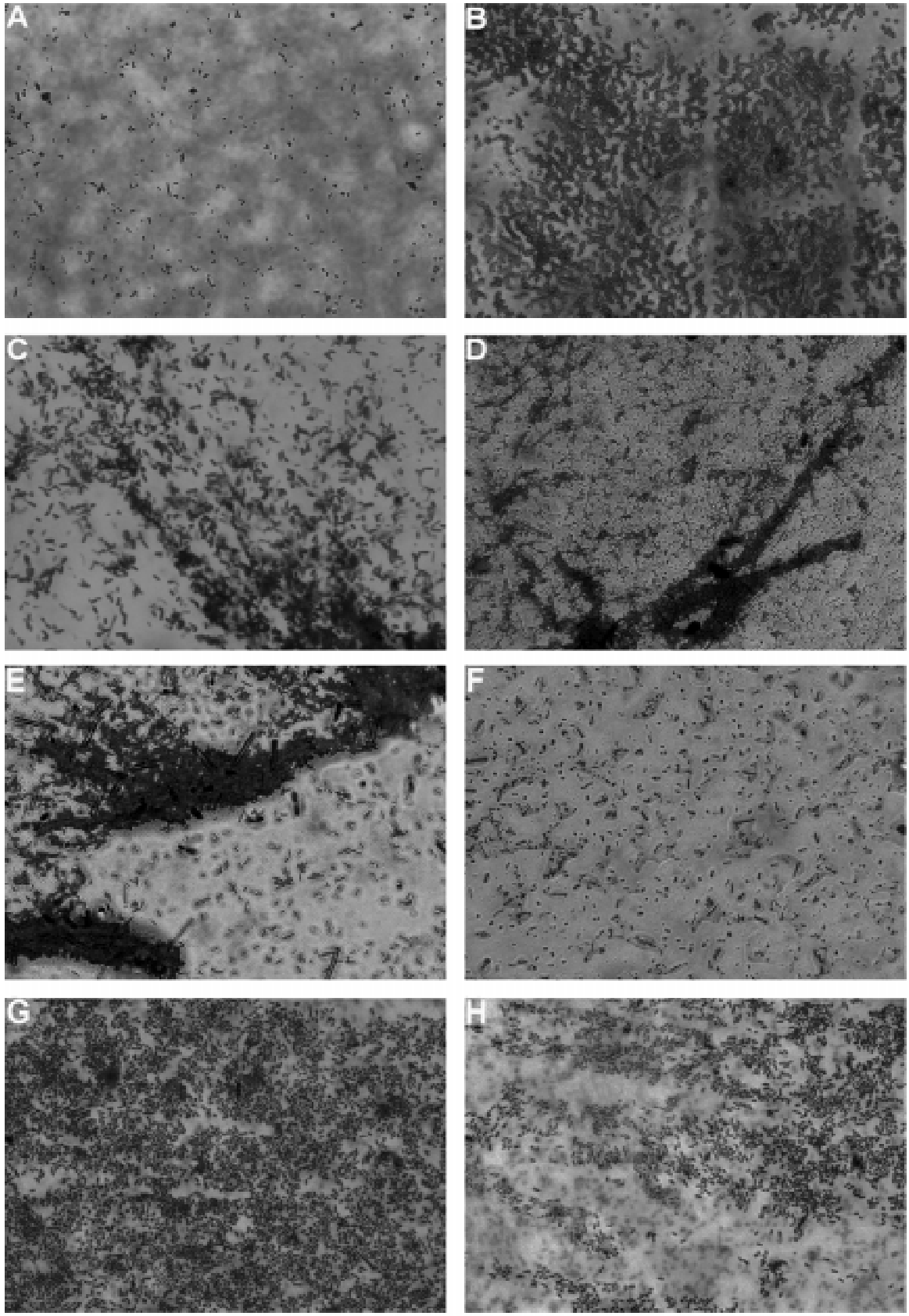

Fig. 2. Biofilm development on glass surfaces examined by a light microscopy.
E. coli biofilms were formed through growth in LB or glucose-minimal medium (laboratory and clinical *E. coli* strains harboring a *dra* gene cluster and its mutants, respectively) and stained with CV. (A) *E. coli* BL21(DE3); (B) *E. coli* BL21(DE3)-pCC90; (C) *E. coli* BL21(DE3)-pCC90DraDmut; (D) *E. coli* BL21(DE3)-pCC90DraCmut; (E) *E. coli* BL21(DE3)-pCC90D54stop; (F) *E. coli* DR14/*gspD*; (G) *E. coli* IH11128; (H) *E. coli* DR14.

uropathogenic *E. coli* strains (van der Woude *et al*., 1992). A high degree of homology between the regulatory proteins and the presence of the highly homologues regions with the GATC sites of the studied gene clusters can suggest the same mechanism of transcription regulation of the *dra* operon. Because the development of biofilm is strictly associated with attachment of bacterial cells to the abiotic or biological surfaces (using surface exposed adhesive organelles) we could not exhibit the formation of bacterial communities by clinical *E. coli* IH11128 strain not expressing Dr fimbriae in rich LB medium (transition from phase ON to phase OFF and abrogation of the *dra* transcription). On the contrary the laboratory *E. coli* BL21(DE3) strains transformed with the plasmids encoding the *dra* gene cluster, without the *draF-draA* regulatory region, and its mutants with an inactivated *draE* or *draC* (the DraE negative and DraD positive strains) and *draD* (the DraD negative and DraE positive strain) showed biofilm formation in either LB and glucose-minimal medium after staining the cells with CV. Furthermore, both DraE and DraD were shown to be required for adherence to abiotic surfaces under static growth conditions. The laboratory *E. coli* strain deficient in the production of the DraD protein was able to form a biofilm but the strains deficient in the surface secretion or production of the DraE showed a greater capacity for this process. The observed differences in development of biofilm were not so clearly visible for clinical *E.* coli IH1128 and DR14 which can suggest a role of another factor besides the DraE and DraD stimulating the bacterial aggregations within urinary tract. The results obtained were confirmed by the light microscopy of laboratory and clinical *E. coli* strains harboring the *dra* gene cluster. Cells attached to the abiotic surfaces were observed as clusters containing cells which were in physical contact, sometimes forming interspersed layers.

On a basis of the investigations performed we can hypothesize that Dr fimbriae capped with the DraD can be critical for the biofilm development as a resistant barrier against hydrodynamic environment of urinary tract. However, the role of DraD in this process is also very significant. Earlier examinations showed that the bacteria exposing at the surface Dr fimbriae with or without the DraD as a tip subunit were able to stimulate a strict adhesion to Hela cells surrounding their whole surfaces (Zalewska-Piątek *et al*., 2008). On the contrary the surface expression of DraD without Dr fimbriae induced aggregation of bacterial cells often adjacent in certain parts of HeLa cells which can suggest the effect of compensation of lack of Dr fimbriae at the cell surface. The same results were obtained by analysis of polystyrene beads coated with DraD or Dr fimbriae (Zalewska-Piątek *et al*., 2008). Therefore, it is possible to assume that the adhesion of *E. coli* Dr^+ strains to host receptors located at the surface of epithelial cells of upper urinary tract mediates the invasion resulting in the formation of bacterial communities by intracellular interactions with the participation of DraD protein. Further studies are needed to determine the exactly role of Dr fimbriae and DraD of uropathogenic *E. coli* strains in biofilm development.

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