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STIMULATION OF SESSILE GROWTH OF A BIOFILM-DEFICIENT STRAIN *ESCHERICHIA COLI* K-12 BY PROTEIN(S) SECRETED BY ENTEROBACTERIACEAE SPECIES

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ABSTRACT

In nature, most microorganisms live associated with a surface and interact with each other, thus forming structures called bioflms. They are often represented by mixed consortia of species and strains among which interaction via cell-to-cell contacts and metabolic secretion occurs. The present study examines the effects of conditioned cell-free culture supernatants (Sp) from three stationary-phase Enterobacteriaceae broth cultures on biofilm growth by the E. coli K-12 strain 446. When grown on 96-well microtitre plates in defined salt medium containing 0.04 M glucose for 24 hours, this strain formed only insignificant amount of biofilm. The supplementation of the medium with Sp from another E. coli K-12 strain did not change this. However, when Sp-s from pathogenic strains (E. coli O157 and Yersinia enterocolitica O3) were added, sessile biomass was significantly increased. Confocal laser scanning microscopy revealed better substratum coverage. The contribution of protein factors and secreted polysaccharides was examined. Treatment with proteinase K reduced biofilm stimulation of the Sp-s. Released polysaccharides were precipitated from the Sp-s, and when applied alone during biofilm growth they showed no significant influence on biofilm development. This indicates a role of protein(s) as biofilm-promoting factor(s).

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Introduction

In most natural environments bacteria associate with a surface and form structures known as biofilms (6). The complicated inter-strain and inter-species interactions that exist in biofilms are of great interest. These involve both cellular contacts (2) and modulation by substances secreted in the environment (9, 12). A variety of secreted factors have been suggested to interfere with biofilm growth among which exopolysaccharides, proteins/peptides, etc. (1, 4, 5, 7, 10, 11, 13). There is less information about the capacity of secreted metabolites to influence the biofilm proficiency of a strain that is uncapable to form biofilm under given conditions. As one of only a few examples, secreted factor(s) available in the cell-free supernatant (Sp) from *E. coli* strain Souza 297 that forms biofilm in the lack of oxygen conferred to other *E. coli* strains the capacity to form biofilms under anaerobic conditions (3).

The present study provides evidence that stationary-phase Sp-s from two pathogenic Enterobacteriaceae strains (*E. coli* O157:H-, and *Yersinia enterocolitica* O:3) can stimulate biofilm growth of the biofilm-deficient *E. coli* K-12 strain 446. The contribution of protein factor(s) and secreted polysaccharides to the biofilm-modulating activities was tested.

Materials and Methods

Strains, media and cultivation

The strain examined for biofilm growth was *E. coli* K-12 446, 33W1485 F^+ lac^+ (NBIMCC, Sofia, Bulgaria). It was stored frozen in Trypticase soy broth (TSB) (Difco) supplemented with 30% glycerol. Before use, samples were inoculated into TSB and incubated overnight at 37 °C. Then bacteria were streaked on nutrient agar for single colony isolation. In the tests of biofilm formation of *E. coli* 446, three single colonies were selected, applied separately to slunted trypticase soy agar, incubated overnight at 37°C and kept refrigerated until use but no longer than 30 days. Samples from these were examined in parallel in the biofilm experiments.

Stationary phase Sp-s were prepared from 48 h TSB cultures of *E. coli* K-12 W1655, F^+ lac^+ Str-s Met- (Prof. J. Gumpert, Institute of Molecular Biotechnology, Jena, Germany) (14, 15) (Sp K-12), *E. coli* O157:H- A2CK SS, Stx1- Stx2- (NCIPD, Sofia, Bulgaria) (Sp O157) and *Y. enterocolitica* O:3 (NCIPD, Sofia, Bulgaria) (Sp Y). The strains were maintained as above, then cultivated in TSB for 48 h at 37 °C. The cells were pelletted for 10 min, 10000 rpm, and the Sp-s were filter-sterilized (0.2 μm, Minisart, Goettingen, Germany). Sterility of the resulting samples was tested in TSB and thyoglycollate broth. The filtrates were stored fozen at –20 °C until use.

Crystal violet (CV) assay for biofilm growth

E. coli biofilm formation was estimated after cultivation in pure M63 medium (0.02 M KH_2PO_4, 0.04 M K_2HPO_4, 0.02 M $(NH_4)_2SO_4$, 0.1 mM $MgSO_4$ and 0.04 M glucose) or M63 medium supplemented with 16%, 8%, or 4% (vol/vol) of the Sp-s. Biofilms were developed on 96-well U-shaped plates for microtitration (Nunc). The wells were inoculated with 10 µl of overnight TSB cultures of the *E. coli* strains (approximately 6×10^5 CFU·ml^{-1} as shown by plating) and cultivated for 24 h at 20 ºC in M63 without (control) or with the Sp-s (100 µl per well). Total bacterial growth was estimated by measuring turbidity. Planktonic cells were removed, the wells were washed 3 times in 0.85% NaCl and the biofilms were stained with 0.1% crystal violet for 10 min. The dye was solubilised with 150 µl 75% ethanol per well and the absorbance was measured at 550 nm. Each variant of the diluted Sp-s was applied in 6 wells per experiment. The data were processed by variation statistics using the Student-Fisher test.

Fluorescence Live-Dead test

Biofilms were cultivated for 24 h at room temperature on microscope cover glasses coated with methyl methacrylate. This was done in Petri dishes containing 6 ml of M63 medium with or without Sp supplements (16%) to which 600 µl of overnight *E. coli* culture in TSB was added. To check the vitality of cells, the plankton was removed and biofilms were colored by L13152 Live/Dead BacLight bacterial viability kit (Molecular Probes) using the protocol recommended by the provider. After 15 min incubation in the dye mixture, the samples were washed in saline and briefly rinsed in sterile distilled water. Then they were left to dry for a short time and mounted face-down on microscopic slides using BacLight™ mounting oil. The observations were made on inverted confocal laser scanning microscope (CLSM) Nikon Eclipse Ti-U.

Proteinase K treatment of supernatants

To check whether the biofilm-modulating activities were of protein nature, 10 µg/ml of Proteinase K (Boeringer anheim Gmbh, Manheim, Germany) was added to the Sp-s. After incubation for 1 h at 37ºC, the enzyme was inactivated for 10 min at 70 °C. The treated Sp-s (16%) (v/v) were added to M63 medium and used for biofilm cultivation as above. Two controls were included: 1) M63 without supplements, and 2) M63 supplemented with 16% (v/v) of the untreated Sp-s.

Isolation and application of crude secreted polysaccharide from the supernatants

Crude polysaccharide was precipitated from 10 ml of each Sp with 100 ml of cold ethanol, centrifuged, resuspended in M63 medium and extensively dialysed against glucose-free M63 medium using dialysis tubing, 12 000 DA cut-off. The amount of sugars in the dialysate was evaluated by the method of Dubois (8). To check for the total amount of secreted polysaccharide in the initial Sp-s, 2 ml of each of them was similarly dialysed and evaluated. For biofilm trials, the isolated polysaccharides from each sample were dissolved in glucose-free M63 medium, and then adjusted to 0.04 M glucose in a way that the final concentration of the secreted polysaccharide was identical to the amount in the 16% initial Sp-s. The thus prepared media were used for biofilm growth. Biofilm was parallelly grown in M63 supplemented with 16% of the initial SP-s. Controls comprised biofilms grown in pure M63.

Results and Discussion

We have previously used the CV test to characterize the biofilm formation capacities of a laboratory collection of eight *E. coli* K-12 strains. Under the experimental conditions applied (24 h growth at 20 °C in defined M63 medium containing 0.04 M glucose), five of these strains were characterized as poor biofilm formers with registered amounts of A_{550} less than 0.05. Stimulation of biofilm growth by metabolites secreted from other bacteria was registered in two of them among which *E. coli* 446 (16).

In the present study, we examined in detail biofilm modulation by Sp-s in *E. coli* 446. Supplementation of the M63 medium with different amounts of Sp K-12 did not result in statistically significant biofilm biomass as registered by the absorbance at 550 nm after CV staining. However, pronounced statistically significant, concentration-dependent biofilm stimulation by the Sp-s from the two pathogenic strains, Sp O157 and Sp Y, was demonstrated (**Fig. 1**).

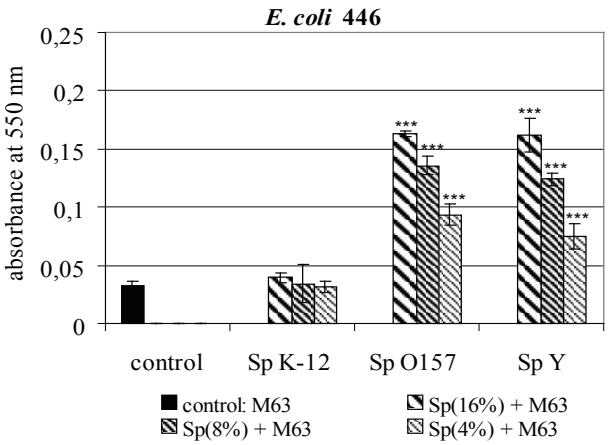

Fig. 1. Effects of the Sp-s on biofilm formation of *E. coli* 446. Biofilm was cultivated in M63 medium supplemented with 16%, 8%, or 4% of the examined Sp-s, and was estimated by the crystal violet assay. The results are the means of at least three independent experiments. Each experiment included 6 repeats per variant. Statistically significant differences from the control (biofilm grown in M63 without supplements) are presented as * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

The results from the CLSM examination coincide well with these results. In spite of the low reactivity in the CV test, some 'isles' of biofilm were observed in control samples and after supplementation with Sp K-12. In these microcolonies, the individual cells were distinct from one another (**Fig. 2 A, B**). Most of the cells were green due to coloring by the Cyto

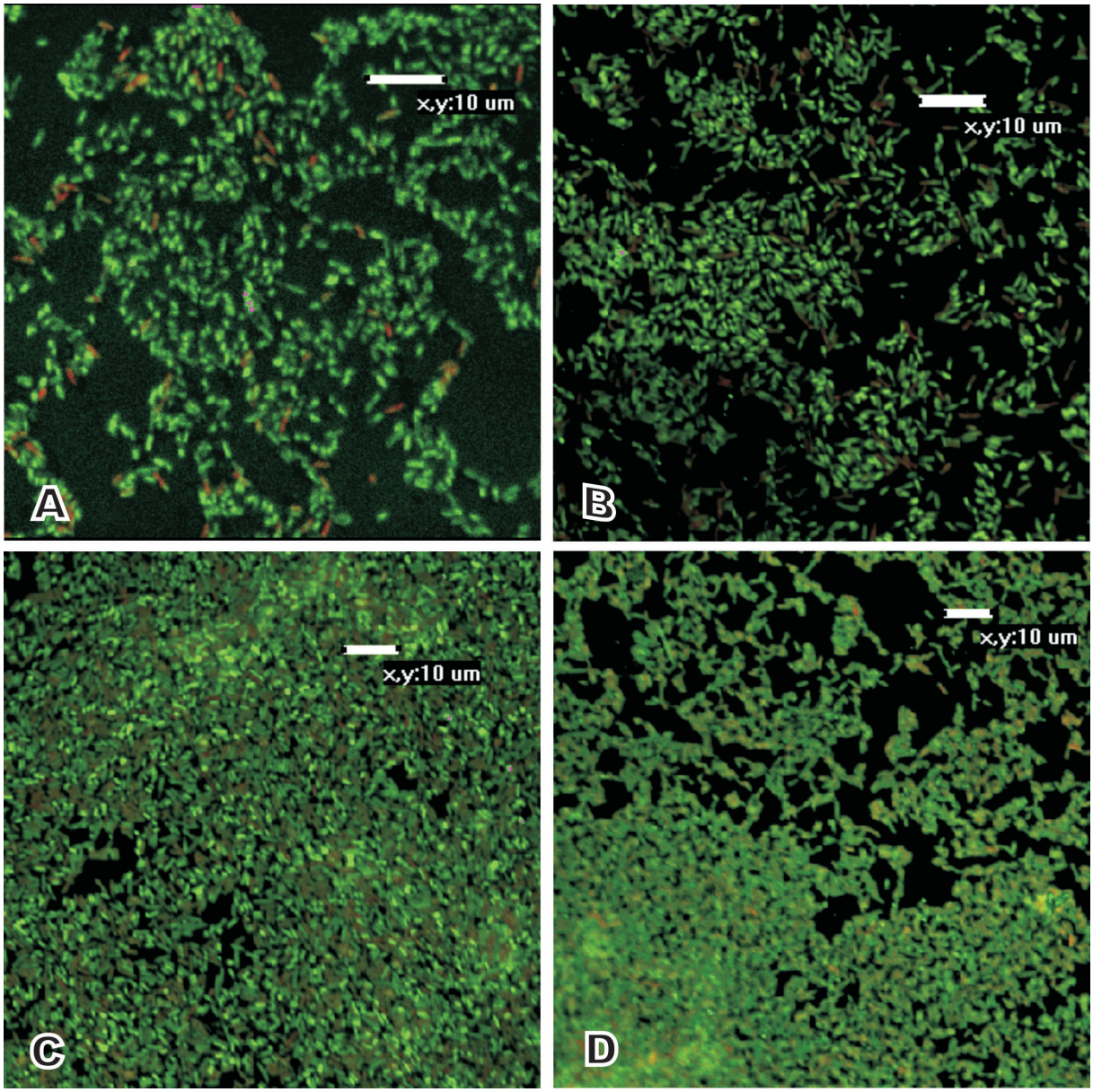

Fig. 2. CLSM of 24 h *E. coli* 446 biofilms grown in the absence (**A**) or presence of 16% Sp K-12 (**B**), Sp O157 (**C**), and Sp Y (**D**). Biofilm was cultivated on cover glasses for microscopy, coated with methyl methacrylate. The samples were stained by fluorescence Live/Dead BacLight bacterial viability kit. Green fluorescence was produced by Cyto 9® that colors all cells, and red fluorescence – by propidium iodide that penetrates in dead cells only. Bar = 10 μm.

component of the dual stain. However red cells also occurred due to propidium iodide staining which labels only dead cells. The biofilms grown in the presence of Sp O157 and Sp Y had significantly higher substratum coverage (**Fig. 2 C, D**). The outline of individual cells was not as distinct as in the first two samples probably due to absorbtion of the dye to DNA fragments present in the extracellular matrix. No visual change in the proportion of live and dead cells was registered.

The above described biofilm stimulation raised the question of what the nature of the active substance(s) in the Sp-s is. It is known that a variety of factors are involved in biofilm formation of *E. coli* among which released exopolysaccharides, some proteins/peptides, signals etc. (1, 4, 5, 7, 10, 11, 13). In our study, the contribution of protein factor(s) and secreted polysaccharides to the stimulating effect of the Sp-s was examined. The treatment of the Sp-s from the two pathogenic strains with 10 μg/ml proteinase K reduced their ability to increase biofilm formation of *E. coli* 446 (**Fig. 3**). These data show that some kind of protein or peptide is one of the factors contributing to the Sp-s effects. The released polysaccharides were applied in amounts approximately similar to their quantities in the untreated supernatants. They showed no

significant influence on biofilm development when compared with the control (pure M63) (**Fig. 3**).

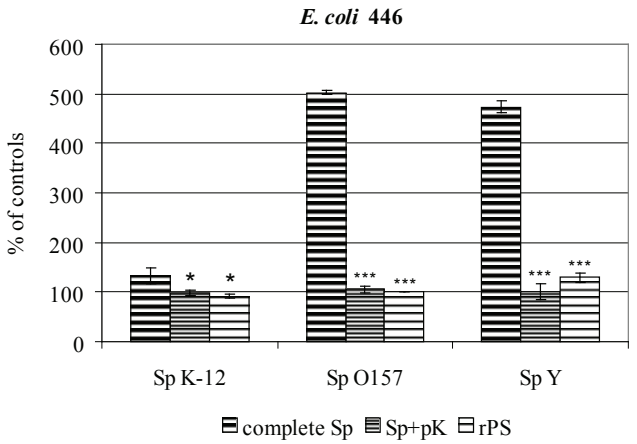

Fig. 3. Effects of proteinase K treatment (labeled "Sp+pK") and of the isolated released polysaccharides (labeled "rPS") on the biofilm-modulation activities of the examined Sp-s applied to *E. coli* 446. Proteinase K was tested as 16% in M63. The quantity of rPS added to M63 was adjusted to equal the estimated amount of secreted polysaccharides in each of the Sp-s. The untreated Sp-s are labeled "complete Sp". Statistically significant differences in biofilm modulation due to the effect of pK and the rPS as compared to the effect of complete Sp-s are labeled: * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$).

Conclusions

Intestinal microorganisms are believed to form biofilm-like structures close to the host epithelia. The commensal bacteria of the gut microbiota represent the regular background against which pathogens have to get established. Laboratory *E. coli* K-12 strains generally originated from commensal faecal isolates. The presently confirmed stimulation of biofilm growth in one such strain in the presence of proteins secreted from pathogenic bacteria might illustrate an evolutionary settled competition mechanism, attempting protection of the host from pathogens by its non-pathogenic inhabitants.

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