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EXPERIMENTAL ARTICLES

Formation of *Pectobacterium carotovorum* Biofilms Depending of the Carbon Source

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Abstract—Microbial biofilms are a basic form of existence of bacteria in the environment, as well as in the animal and plant organisms. The patterns of biofilm formation depending on cultivation conditions is presently insufficiently studied. This paper presents experimental results on the effect of carbon sources on biofilm formation and movement on the swarming type in a phytopathogenic bacterium *Pectobacterium carotovorum*. A polyol inositol was shown to cause a significant activation of these processes.

Keywords: biofilms, *Pectobacterium carotovorum*, inositol, swarming
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While ability of microorganisms to form aggregations termed biofilms has been known for over 30 years (Costerton et al., 1987), it still attracts attention of researchers due to almost ubiquitous occurrence of biofilms and their important role in many fields.

Transition from planktonic existence to life in a multicellular community results in improved cell survival under unfavorable conditions (Oliveira, 2015). Biofilm formation depends on temperature, medium composition, density of a bacterial population, presence of other organisms, etc. This process is stimulated by antibiotics, heavy metals, and plant flavonoids (Olsen, 2015). Efficient methods for controlling this transition are presently absent due to its complex, multistage regulation. The works on assessment of the effect of environmental factors on biofilm formation are therefore of importance.

The goal of the present work was to determine the effect of the carbon source added to starvation medium (phosphate buffer saline, PBS) on biofilm formation by *Pectobacterium carotovorum* VKM B-1247.

MATERIALS AND METHODS

The subject of investigation was a plant pathogenic bacterium *Pectobacterium carotovorum* VKM B-1247, an agent of soft rot causing cell wall degradation in a number of plants. The strain was obtained from the All-Russian Collection of Microorganisms (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences). Bacteria were grown on meat infusion agar (NITsF, St.-Petersburg, Russia).

Biofilm formation was studied using the phosphate buffer saline (PBS) containing the following (g/L): Na_2HPO_4, 0.058; KH_2PO_4, 0.035; NaCl, 8.5; pH 7.0. Sugars (glucose, fructose, xylose, ribose, lactose, or maltose) and polyols (mannitol, dulcitol, or *myo*-inositol) were added to PBS as the sole carbon source (5 g/L).

The 24-h culture was washed off the agar medium with PBS. The medium (50 mL PBS with a carbon source) was inoculated with 1.5 mL of bacterial suspension (OD_{540} 0.26–0.33), mixed, and dispensed into sterile 96-well plates (150 μL per well). Optical density of the suspension was measured using an Infinite 200 plate reader (Tecan, Switzerland) 3, 5, and 7 days after inoculation. The medium (PBS with the relevant sugar) was used as the control. For quantitative analysis of biofilms, planktonic cells were removed and attached ones were stained with 1% crystal violet. The bound stain was extracted with ethanol, and OD_{540} of the solution was determined (Shaginyan et al., 2007).

Visualization of the biofilms on cover slides was carried out under an Axio Observer Z1 microscope (Carl Zeiss, Germany). Sterile fat-free slides were incubated in petri dishes with 30 mL of the medium (PBS with a carbon source) for 3 and 8 days. The slides were then washed to remove weakly attached cells, stained with

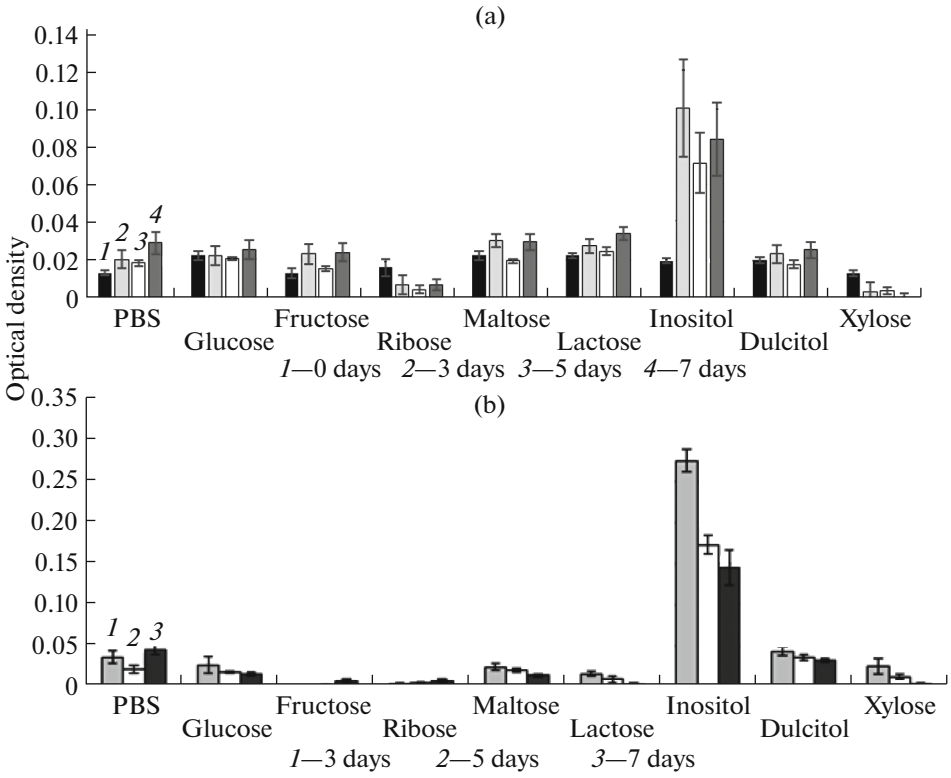

Fig. 1. Optical density of *P. carotovorum* cell suspension (a) and stained biofilm (b) in media with different carbon sources.

1% crystal violet, and examined at ×100. For each sample, cell number and size were determined in ten fields of view using the Axio Vision software package.

The effect of *myo*-inositol (Serva, Germany) on biofilm formation was studied using 12 twofold serial dilutions in PBS with the initial concentration of 5 g/L (0.5%).

Effect of the carbon source on bacterial swarming motility was studied in nutrient broth or PBS with 0.6% agar and 5 g/L glucose or *myo*-inositol (Kearns, 2010). Microbial suspension with OD_{540} 0.26–0.33 (10 μL) was applied to the agar surface. The colony size was measured after seven days of incubation.

Statistical treatment. All experiments were carried out in at least three replicates. The R programming environment v. 3.10 was used for statistical treatment of the data (Zaryadov, 2010). Occurrence of deviations in the samples was determined using the Dickson's Q test. Their compliance with the Gauss distribution was assessed using the Shapiro–Wilk criterion. Significance of the results was determined by single-factor dispersion analysis using the nonparametric Kruskal–Wallis test. Cluster analysis was carried out using the Word method (Oksanen, 2014).

RESULTS AND DISCUSSION

Effect of the carbon source on biofilm formation was studied in PBS, a starvation medium unfavorable for microbial growth. We have previously reported survival of a number of heterotrophic bacteria in this medium (Markova et al., 2013). We suggested activity of the toxin–antitoxin systems, which under unfavorable conditions induce death of some cells in the population, thus providing nutrients for the remaining cells (Yamaguchi et al., 2011). This is, however, not accompanied by biofilm formation, which is an energy-consuming process which probably does not occur under extreme starvation conditions.

In the present work all tested carbon sources, except for *myo*-inositol, were found to have no significant stimulatory effect on *Pectobacterium carotovorum* VKM B-1247 growth in both planktonic and biofilm cultures (Fig. 1).

Importantly, glucose did not stimulate the growth of the planktonic population. This sugar is known to be the preferred nutrient substrate for enterobacteria (Fig. 1).

Light microscopic visualization of *P. carotovorum* cells sorbed on glass slides revealed adhesion of individual cells after one day of cultivation in PBS, while small cell aggregates were observed after eight days (Fig. 2).

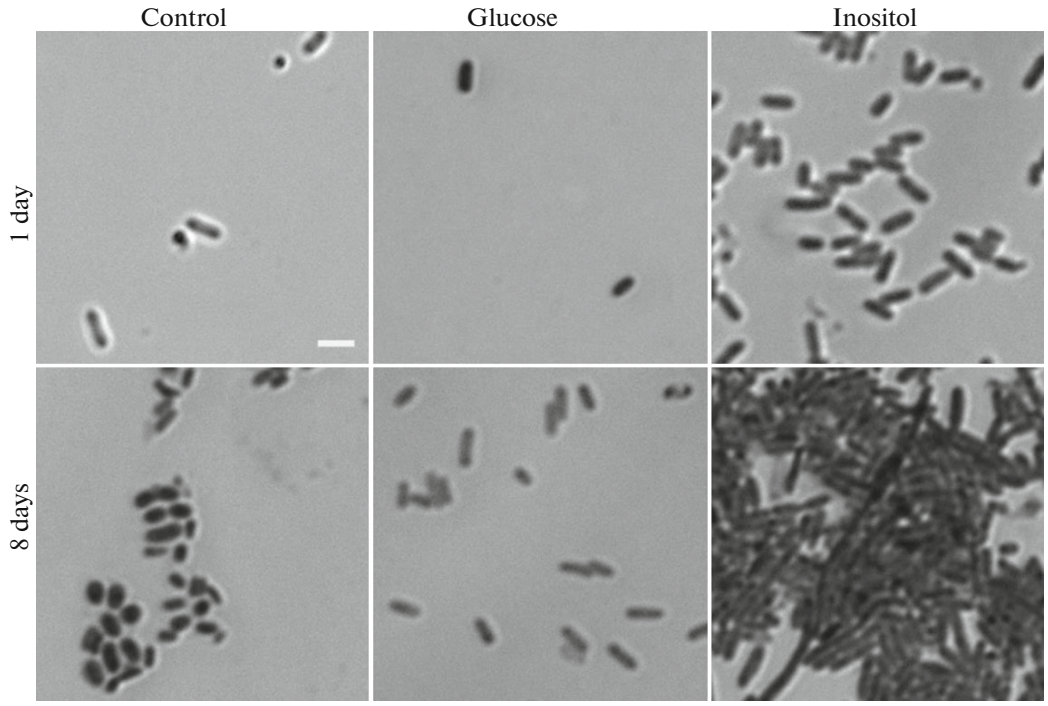

Fig. 2. *P. carotovorum* biofilms. Scale bar, 5 μm.

No biofilm formation occurred during growth in PBS with glucose, which was in agreement with the results of spectrophotometric analysis (Fig. 1b). Among other substrates, only *myo*-inositol provided for formation of significant numbers of sorbed cells and of biofilm-like multicellular structures. These results correlated with the data obtained by spectrophotometry. The average number of sorbed cells was calculated for each substrate. The results are presented in Table 1.

Microscopy revealed another feature of the cells, namely variations in cell size depending on cultivation conditions. The cells formed in PBS after eight days were of a rounded shape typical of the stationary-phase cells, as can be seen from their length-to-width ratios (Table 2). Unfavorable conditions caused by nutrient limitation are know to affect cell shape (Somova et al., 2009). In enterobacteria this transition is associated with activity of the *bolA*, expression of which results in spherical cell shape (Santos, 2002). This gene is induced under stress conditions, including carbon limitation.

In eight-day cultures growing in liquid media with glucose or *myo*-inositol the cells were more elongated, especially in the case of *myo*-inositol. This is probably due to the properties of swarmer cells acquired by *P. carotovorum* cells. Such morphology is acquired in the course of transition to swarming motility. Similar to biofilm formation, swarming is considered a spatially organized form of bacterial existence. While biofilms are attached structures, swarming makes it possible for a microbial population to move in coordination over or inside a substrate. In this state the cells are elongated (Kearns, 2010). In laboratory conditions swarming is studied in semisolid agar. While bacteria form colonies on the surface of solid agar media, at lower agar concentrations (0.4–0.6%) microbial cells are able to migrate using their flagella.

Effect of *myo*-inositol on *P. carotovorum* swarming motility was studied in two media, full-strength one based on meat infusion broth and PBS-based starvation medium. The experiments were carried out in

Table 1. Numbers of *P. carotovorum* (cells/cm^2) sorbed on glass in the course of incubation in phosphate buffer saline with different carbon sources (cells counted in ten fields of view using the Axio Vision software package)

Carbon source	Cultivation time, days		
	1	6	8
None	19 ± 2	0	129 ± 52
Glucose	12 ± 2	0	7 ± 9
Xylose	17 ± 3	40 ± 8	47 ± 20
Fructose	82 ± 21	815 ± 223	6 ± 2
Dulcitol	0	16 ± 4	31 ± 3
Maltose	13 ± 1	66 ± 12	53 ± 13
Lactose	5 ± 14	9 ± 1	20 ± 3
Ribose	0	0	7 ± 1
Inositol	365 ± 6	915 ± 53	3529 ± 177

Table 2. Size of *P. carotovorum* cells sorbed on glass in the presence of different carbon sources

Carbon source	Incubation time, days	Cell length, μm	Cell width, μm	Length-to-width ratio
None	1	1.73 ± 013	0.91 ± 0.05	1.90
	8	1.62 ± 0.11	1.01 ± 0.05	1.61
Glucose	1	1.52 ± 0.10	0.64 ± 0.02	2.37
	8	2.01 ± 0.12	0.83 ± 0.04	2.42
Inositol	1	2.01 ± 0.09	0.92 ± 0.03	2.19
	8	2.47 ± 0.13	0.70 ± 0.02	3.52

three variants: without carbohydrates, with glucose, and with inositol. It was found that *myo*-inositol promoted swarming, especially in starvation medium (Fig. 3).

Thus, addition to starvation medium of *myo*-inositol as the sole carbon source facilitated transition of *P. carotovorum* cells to a spatially organized mode of existence, favoring both biofilm formation and swarming motility.

Biofilm formation by *P. carotovorum* at different *myo*-inositol concentrations was studied at the next stage of the work. Staining of bacterial biofilms with crystal violet revealed that the effect of *myo*-inositol increased with its increasing concentration. Cluster analysis of the results revealed that all experimental variants fell into several groups, i.e., the group with the highest inositol concentration (5 g/L) and the remaining ones, which in turn formed three clusters: the one with 2.5 g/L inositol (a twofold dilution), the one with 3- to 5-fold dilutions, and the one with 6- to 12-fold dilutions. The control variant (PBS) fell into the latter cluster (Fig. 4).

The effect of a carbon source on formation and dispersion of bacterial biofilms is presently insufficiently understood. In theory, glucose, which is transported into the cell via a phosphoenolpyruvate-dependent phosphotransferase system (PTS), is a preferred carbon source for most microbial species and should inhibit transition of planktonic cells to an attached state due to the absence of stress impacts. Thus, investigation of biofilm formation by *Streptococcus mutans* demonstrated expression of the genes involved in these

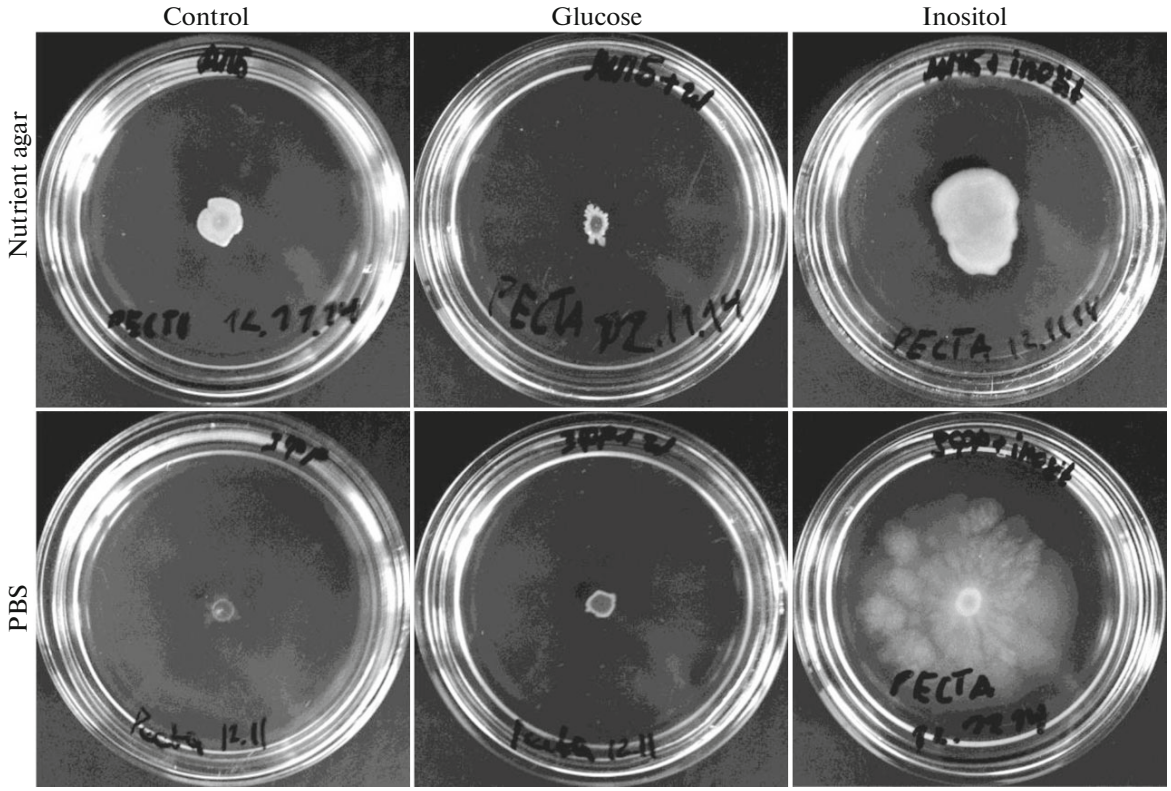

Fig. 3. Swarming of *P. carotovorum* cells on different nutrient media.

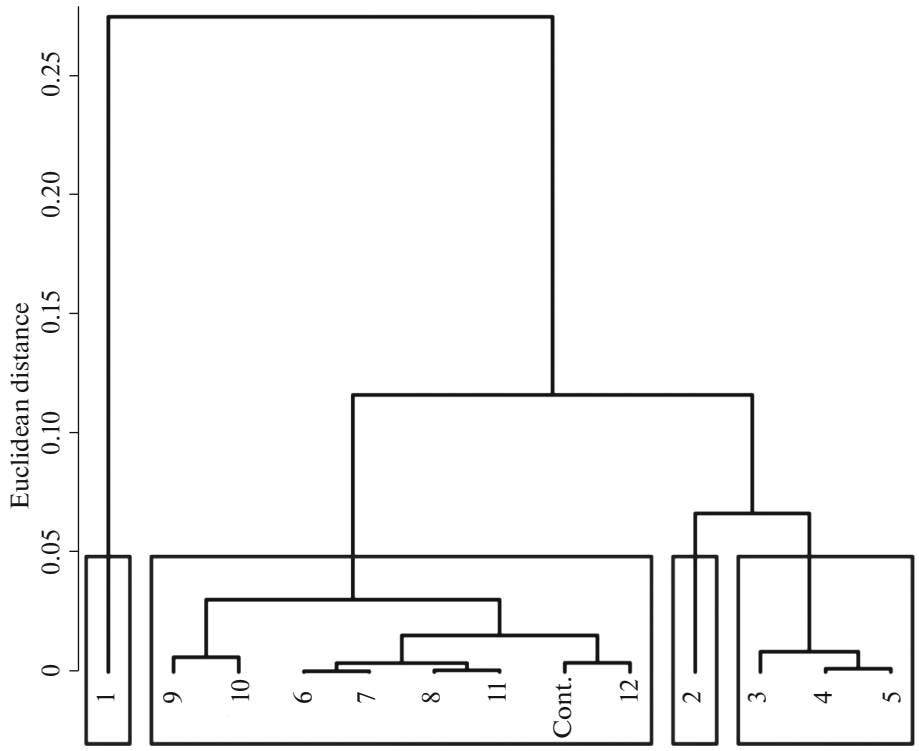

Fig. 4. Cluster dendrogram of biofilm formation by *P. carotovorum* at different inositol concentrations in the medium: control (phosphate buffer saline without carbon sources) and inositol concentrations, %: 0.5 (1), 0.25 (2), 0.125 (3), 0.063 (4), 0.031 (5), 0.016 (6), 0.008 (7), 0.004 (8), 0.002 (9), 0.00098 (10), 0.00049 (11), and 0.00024 (12).

processes to be inhibited by glucose (Shemesh et al., 2007). However, both inhibitory and stimulatory effects of glucose on biofilm formation have been reported. Response to this carbohydrate may be different in different microbial species. The concentration and, as our work showed, the basal medium to which a carbohydrate is added (starvation medium, broth, etc.) are also important factors (Kim and Frank, 1995; Korobov et al., 2010; Waldrop et al., 2014; Ran et al., 2015).

Other carbohydrates and polyols are known to affect this process. Addition of xylitol to the medium for cultivation of *Streptococcus pneumoniae* was found to inhibit biofilm formation, while no inhibitory effect of xylitol on biofilm formation was observed in the presence of glucose or fructose (Kurola et al., 2011). Other authors reported combined action of lactoferrin and xylitol to impair the structure of *Pseudomonas aeruginosa* biofilms resulting in decreased survival of microbial cells (Ammons et al., 2009).

Biofilm formation by *Streptococcus mutans* was induced by addition of lactose to the medium. Interestingly, this sugar exhibited no effect on the growth of planktonic forms of this microorganism, thus having a specific effect on biofilm formation. Exopolysaccharides (EPS) produced by lactose-grown *S. mutans* differed from the EPS formed during growth on sucrose (Assaf et al., 2015). Mannose and trehalose stimulated biofilm formation by *Listeria monocytogenes* (Kim and Frank, 1995). Enriching the growh medium with galactose (0.1–1.0%) or mannose (0.5–1.0%) resulted in a significantly decreased biofilm formation by *Staphylococcus epidermidis* 33 (Korobov et al., 2010). Thus, dependence of biofilm formation on the presence of a carbon source in the medium is different for different bacterial species. It may be determined by such factors as the density of a microbial population, its physiological age, the presence of other biogenic elements, etc.

Inositol (cyclohexane-1,2,3,4,5,6-hexol), a sixfold alcohol (polyol) of cyclohexane. It exists in nine possible stereoisomers, of which the most prominent form, widely occurring in nature, is *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol, or *myo*-inositol. It occurs in soil, plants, and animals (Rothhardt, 2014). A number of microorganisms, including *Bacillus subtilis*, *Cryptococcus melibiose*, *Aerobacter aerogenes*, *Rhizobium leguminosarum* bv. *viciae*, *Sinorhizobium meliloti*, *Sinorhizobium fredii*, *Corynebacterium glutamicum*, and *Lactobacillus casei,* may grow on *myo*-inositol as the sole carbon source (Kohler et al., 2010). The pathways of its catabolism to acetyl-CoA and dihydroxyacetone phosphate in *B. subtilis* were studied. In this bacterium, the genes of the inositol utilization pathways are located in two operons, *iolABCDEFGHIJ* and *iolRS* (Yoshida et al., 2008).

In gram-negative bacteria, the genes and enzymes involved in *myo*-inositol utilization are poorly studied. They are localized on a 22.6-kb island (Rothhardt, 2014). Among enterobacteria, their presence was shown for *Erwinia*, *Pectobacterium*, *Citrobacter*, and *Yersinia* strains (Kröger and Fuchs, 2009). Regulation of the *iol* genes is carried out by the IolR repressor, which binds the promoters of these genes in a rich medium. The ReiD protein activating transcription of the *iolE* and *iolG1* genes involved in the first stage of *myo*-inositol degradation was recently identified. This stage results in formation of an intermediate, 5-keto-2-deoxy-D-gluconic acid 6-phosphate, which binds with the IolR protein causing its dissociation from the *iol* promoters (Rothhardt, 2014).

As a component of phosphoinositides, inositol plays an important part in the regulation of dynamic membrane modifications and intracellular signaling in eukaryotes (Morita et al., 2010). This signaling pathway was not observed in prokaryotes. Some species of actinomycetes and archaea were, however, shown to be able to synthesize a phosphoinositide phosphatidylinositol-3-phosphate (Morita et al., 2010). The enzymes phosphatidylinositol phosphate synthase (PIP) and archaetidylinositol phosphate synthase (AIP) were found in archaea and eubacteria (Morii et al., 2014). Existence of this pathway for signal transmission in some prokaryotic species may therefore be suggested.

Our experiments demonstrated convincingly that *myo*-inositol promotes transition of *P. carotovorum* cells to the biofilm mode of existence. The results do not indicate the signal effect of *myo*-inositol, since almost no inductive effect was observed at its low concentrations in the medium. Products of inositol metabolism, rather than inositol proper, may be inducers of biofilm formation. The results obtained certainly indicate necessity for further research.

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