Pluronics' influence on pseudomonad biofilm and phenazine production

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Abstract

Colonization of roots by Pseudomonas chlororaphis O6 (PcO6) involves root surface coverage through surface motility and biofilm formation. Root colonization and the production of antifungal phenazines are important in the ability of the bacterium to protect plants against pathogens. In this in vitro study we report that both biofilm formation and phenazine production are differentially influenced by nutrition and the presence of polyethylene oxide/polypropylene oxide triblock copolymer surfactants (Pluronics). Such surfactants are used for many purposes including agricultural formulations. Four Pluronics differing in molecular weight and in hydrophobic/hydrophilic proportions had distinct effects on biofilm formation and secondary metabolite production, although each increased surface motility, termed swarming, to a similar extent. These findings show that Pluronics had specific metabolic impacts on the bacterium, where both up- and downregulation was achieved depending on the medium and the Pluronic composition. In environmental and agricultural settings, Pluronics may have unanticipated effects on soil microorganisms, while in bioprocessing these effects may be leveraged to regulate metabolite yield.

Introduction

Pluronics (poloxamers) are nonionic copolymers containing hydrophobic polypropylene oxide (PPO) and hydrophilic polyethylene oxide (PEO) blocks arranged as PEO–PPO–PEO for Pluronics and PPO–PEO–PPO for Reverse (R) Pluronics. The hydrophile–lipophile balance dictates polymer amphiphilicity and the resulting surface activity, allowing properties to be tuned to specific applications such as wetting agents, emulsifiers, defoaming agents, and lubricants. Pluronics are used for many purposes (Edens & Whitmarsh, 2004; Fusco et al., 2006) including in formulations for agricultural pesticides (Haas et al., 2001; de Ruiter et al., 2003; Lui et al., 2008) and as a means to reduce bloat in ruminants (Stanford et al., 2001). Thus, exposure of soil bacteria to Pluronics is likely in the environment.

In this study, we examine the effect of four Pluronics (F108, P104, P123, and 25R2) on a soil bacterium, Pseudomonas chlororaphis O6 (PcO6). We assayed three traits important in the survival of this bacterium: biofilm formation, swarming motility, and secondary metabolite production. Both biofilm formation and swarming abilities are traits involved in root colonization (Sánchez-Contreras et al., 2002; Danhorn & Fuqua, 2007; Rudrappa et al., 2008). PcO6 is an aggressive plant root-colonizing bacterium (Spencer et al., 2003) and forms robust biofilms in vitro (Anderson et al., 2005; Narasimhan, 2006). Pluronic activity on PcO6 swarming has not been investigated; however, other bacteria exhibit enhanced swarming in the presence of the nonionic polysorbate surfactant Tween-80 (Niu et al., 2005). PcO6 produces a variety of secondary products important in biocontrol, including yellow- and orange-colored phenazines, hydrogen cyanide, and exoproteases (Spencer et al., 2003).

The Pluronics P104 \([{(EO)_{27}}-(PO)_{61}-(EO)_{27}]\), P123 \([{(EO)_{20}}-(PPO)_{70}-(EO)_{20}]\), and 25R2 \([{(PO)_{22}}-(EO)_{24}-(PO)_{22}]\) were selected from a range of Pluronics based on our prior studies showing metabolic effects on bacteria (Narasimhan, 2006). F108 \([(EO)_{129}-(PO)_{56}-(EO)_{129}]\) is a Pluronic frequently used to decrease bacterial adsorption to surfaces (Gombotz et al., 1991; Costerton et al., 2005). The properties of the Pluronics are summarized in Table 1, and
show that these Pluronics span a range of molecular weights (MWs) and hydrophilic–lipophilic ratios. The reduction in the surface tension of water by the Pluronics is presented as this is an indicator of wetting ability and surface activity. Distinct differences in PcO6 biofilm formation and phenazine production were observed to be both Pluronic- and growth medium-dependent, whereas swarming was enhanced to a similar extent for each Pluronics.

Materials and methods

Bacterial strains and growth media

The wild-type PcO6 strain O6 obtained from the roots of field-grown wheat (Radtke et al., 1994) was maintained frozen at −70 °C in 15% glycerol with cells grown in minimal medium containing in 1L: 10.5 g K2HPO4, 4.5 g KH2PO4, 0.5 g sodium citrate·2H2O, 1.0 g (NH4)2SO4, 0.25 g MgSO4·7H2O, and 2.0 g sucrose. A mutant strain lacking the ability to produce phenazines because of inactivation of the phzA gene by the insertion of a kanamycin-resistance gene was derived as described by Kang et al. (2007). This mutant was grown in minimal medium amended with 20 μg mL⁻¹ kanamycin before preparation of stock cultures. For studies, cells were grown at 23 °C in liquid minimal medium or Luria–Bertani (LB) medium modified by the omission of NaCl.

Biofilm studies

Biofilm formation was studied in 1-mL cultures grown in 12-well polystyrene tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). LB or minimal medium was inoculated using 10⁶ cells directly from glycerol stocks and cultures were grown on a platform rocker moving at three rocker movements per minute at 20 °C. Pluronics F108, P104, P123, and 25R2 (BASF Corporation) were added to the liquid growth media to achieve a final concentration of 0.5% v/v before inoculation. Pluronic is a registered trademark of BASF Corporation. After 6 days, planktonic cells were removed from each well and growth was estimated by measuring turbidity at 600 nm and by dilution plating onto LB agar medium to determine culturable colonies. CFU mL⁻¹ was calculated after 2 days of colony growth. Biofilm formation in the well plates was determined following removal of planktonic cells, and subsequent washing of each well with sterile water twice before treatment with 0.25% (w/v) crystal violet in sterile water. The stained biofilm cells were washed three times with water, and the absorbed crystal violet was extracted into 1 mL ethanol before reading A570 nm (Djordjevic et al., 2002). Statistical evaluation was conducted using statistical software SPSS version 15.

Swarming motility

Swarming motility was examined on minimal medium with 0.5% agar and amended with 0.5% Pluronics as appropriate. An inoculum of 3 μL PcO6 with 10⁶ cells mL⁻¹ was applied to the center of each plate and the plates were incubated at 26 °C for 5 days before the diameter of the colony was measured. Two separate studies were performed, each with two replicates for each condition.

Phenazine production

Phenazines were assessed in the culture medium by centrifuging the cultures from the well plates at 10 000 g for 5 min to precipitate the cells. The absorbance of the culture filtrates was measured spectrophotometrically at 367 nm, which is characteristic of phenazines (Radtke et al., 1994). The findings were replicated by examining phenazine formation in culture filtrates from 50-mL cultures grown in 125-mL flasks for 48 h at 28 °C with shaking at 100 r.p.m. Cells were removed by centrifugation at 10 000 g for 5 min and the supernatant was adjusted to pH 2.0 before mixing (1 : 1, v : v) with benzene (Krishnan et al., 2007). The benzene layer was recovered and dried. Benzene (100 μL) was added and the solution was applied as 10-μL aliquots to thin-layer chromatography (TLC) Silica Gel 60 plates. Chromatography was performed in benzene:glacial acetic acid (95:5, v : v) before evaporation of the solvent in the air and viewing under visible light and UV to observe fluorescence.

Results

Biofilm growth

PcO6 biofilms formed on the bottom and sides of the well plates in both media. Assays after 2–7 days of culture were performed initially and showed that biofilm intensity, as determined by staining with crystal violet and extraction of this dye, increased from 2 to 4 days and was then consistent for 7 days. Data shown are for 6-day studies to attempt to demonstrate any long-term adhesive effects of the Pluronics. Control studies showed that PcO6 did not use the Pluronics as a growth source, in agreement with another bacterial/Pluronic study (Splendiani et al., 2006).

Table 1. Properties of the Pluronics used in the studies

<table>
<thead>
<tr>
<th>Pluronic</th>
<th>MW</th>
<th>%PEO by mass</th>
<th>Surface tension*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P123</td>
<td>5750</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td>P104</td>
<td>5900</td>
<td>40</td>
<td>33</td>
</tr>
<tr>
<td>F108</td>
<td>14 600</td>
<td>80</td>
<td>41</td>
</tr>
<tr>
<td>25R2</td>
<td>3100</td>
<td>20</td>
<td>43</td>
</tr>
</tbody>
</table>

*0.1% v/v in water, dynes cm⁻¹ at 25 °C.
The CFU mL\(^{-1}\) data for the planktonic \(Pc\)O6 cells in the well plate cultures were comparable for both media types, with little effect due to the presence of Pluronics (Table 2). In contrast, biofilm formation was strongly medium dependent and influenced by the selected Pluronics as illustrated in Fig. 1. Enhanced biofilm formation in minimal medium is considered to be an adaptive response to nutrient availability, favoring adherent, sessile bacteria. In the defined minimal medium F108 and 25R2 significantly reduced biofilm formation, while P104 and P123 showed little effect.

### Swarming motility

Initial studies demonstrated that \(Pc\)O6 had a weak swarming motility. Colony diameter after 5 days of growth on 1.5% minimal medium agar was 0.60 (± 0.05) cm compared with 2.3 (± 0.2) cm on 0.5% agar. When the 0.5% agar was amended with Pluronics, each of these surfactants increased the colony diameter compared with growth on plates lacking Pluronics (Fig. 2). There was no statistical difference between the Pluronics (\(P = 0.05\)) in this enhancement of motility.

### Phenazine production

Phenazine production of \(Pc\)O6 was affected by nutrition, with higher levels being produced in the rich LB medium than in the defined minimal medium, as observed in Fig. 3a. \(A_{367}\) nm of cell-free culture filtrate confirmed these visual observations (Table 2). In both media, Pluronic 25R2 intensified the extent of orange coloration and this correlated with the increased intensity of the hydroxylated derivatives shown by TLC analysis (Fig. 3b). In LB medium, reduced orange coloration was noted in cultures amended with P104 and P123. The orange coloration correlated with phenazine formation because studies with a \(Pc\)O6 mutant that does not produce phenazines due to a mutation in \(phzA\) (Kang et al., 2007) revealed no visible changes in orange coloration or \(A_{367}\) nm when grown on a rich medium with

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**Table 2. Effect of Pluronics on growth and phenazine production by *Pseudomonas chlororaphis* O6 in LB and defined minimal medium**

<table>
<thead>
<tr>
<th>Sample</th>
<th>LB medium CFU mL(^{-1}) (\times 10^9)</th>
<th>Minimal medium CFU mL(^{-1}) (\times 10^9)</th>
<th>(A_{367}) nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.9 ± 0.7</td>
<td>1.6 ± 0.3</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>F108</td>
<td>1.9 ± 0.8</td>
<td>1.5 ± 0.5</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>P104</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>P123</td>
<td>2.1 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>25R2</td>
<td>1.4 ± 0.7</td>
<td>2.2 ± 0.2</td>
<td>1.3 ± 0.7</td>
</tr>
</tbody>
</table>

Cells were grown in well plates on rich (LB) medium or on defined minimal medium as described in Materials and methods and dilution plated to determine CFU mL\(^{-1}\). The \(A_{367}\) nm of the cell-free culture filtrate was measured to assess phenazine level. Data are the results of one of three studies each with similar results. The mean of three replicates for one study and the SE are shown.

In LB medium, only P104 exhibited significant biofilm inhibition.

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**Fig. 1.** Pluronic influence on biofilm formation in rich and defined media. Biofilm intensity was determined by crystal violet staining at 570 nm after growth on minimal medium (a) and LB (b). Data are averages of three separate studies, each with three replicates for each treatment. Error bars represent the SD of the mean.

**Fig. 2.** Pluronics increase the swarming motility of \(Pc\)O6. Studies were performed with minimal medium containing 0.5% agar in the presence and absence of Pluronics. Data are the averages of two studies, each with two replicates. Error bars represent the SD of the mean. Letters above the column indicate statistical differences at \(P = 0.05\).

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and without Pluronic additions (data not shown). TLC of the benzene extracts (Fig. 2b) showed PcO6 to produce phenazine-1-carboxylic acid (PCA), 2-hydroxy-PCA (2-OH-PCA), and 2-hydroxy-phenazine (2-OH-phenazine) as published (Radtke et al., 1994; Krishnan et al., 2007). This TLC analysis confirmed the reduction in phenazine production in the presence of P104 and P123 and increase in phenazine production with 25R2. An additional observation (data not shown) was that cells pelleted by centrifugation from 25R2-cultures had a uniform deep-orange color, whereas only a portion of the cells from the control culture were orange colored.

**Discussion**

Pluronic copolymers present in the growth media for the pseudomonad strain PcO6 differentially altered both biofilm and secondary metabolite production. To our knowledge, this is the first study showing that Pluronics differentially affect biofilm formation as a function of the nutrient conditions. It is likely that there are differences in the mechanisms of biofilm formation under the rich and defined media conditions (Anderson et al., 2005; Narasimhan, 2006). Consequently, we predict that these processes were differentially sensitive to the Pluronics. Our finding that F108 decreased biofilm formation in minimal medium agreed with previous studies of Pluronics as nonfouling coatings (Gombotz et al., 1991; Costerton et al., 2005). However, under our test conditions, F108 was not effective in preventing biofilm formation under rich medium growth conditions. These findings suggested that the nutrient conditions may influence the effectiveness of a Pluronic on adhesion.

In contrast to the differential effects of Pluronics on biofilm formation, each Pluronic enhanced swarming of the PcO6 cells. This effect may be related to the wetting properties of each Pluronic (Table 1). Our findings agree with the observation that amendment with the surfactant Tween also enhanced swarming for other bacteria (Niu et al., 2005). Swarming seems to be relatively weak in PcO6 and we have not been able to definitively establish by chemical assays whether this bacterium makes its own surfactant. However, recent studies by Chen et al. (2007) showed that the ‘wetting agent’ produced by a bacterium for swarming motility need not be a surfactant. Our finding that each of the Pluronics similarly enhanced PcO6 surface motility suggested that their differential activity on biofilm formation had some other basis than surfactant activity.

The Pluronics also affected phenazine production differentially, with 25R2 increasing production and P104 and P123 causing decreases and F108 having no effect. We are further investigating the mechanism by which Pluronics alter phenazine production. The hydrophilic–hydrophobic balance and chain lengths of Pluronics define membrane-permeabilizing properties in eukaryotic cells. An altered membrane function has been reported for eukaryotic cells when exposed to Pluronics (Melik-Nubarov et al., 1999; Krylova et al., 2001; Demina et al., 2005). For example,
Pluronic P85 inhibits the P-glycoprotein drug efflux system (Batrakova et al., 2001, 2003; Kabanov et al., 2003). Krylova et al. (2001) proposed that the middle hydrophobic PPO block inserts into the lipid bilayer while the hydrophilic end groups (PEO) disturb the bilayer structure, increasing the membrane permeability. They demonstrated that an increase in the PPO block length of a particular Pluronic improved its ability to enhance the lipid membrane permeability. Of the Pluronics investigated here, 25R2 had the lowest MW, the highest PPO:PEO ratio (80:20), and a PPO–PEO–PPO (i.e. reverse) configuration (Table 1) that may further enhance its membrane-penetrating ability. The two PPO segments of 25R2 may both reside in the outer bilayer, leaving the PEO segment to form a loop in the head-group/lipopolysaccharide region. It is also possible that the reverse structure allows 25R2 to span both lipid bilayers of the bacterium, with the PEO segment residing in the peptidoglycan layer. Altered permeability or intracellular signaling is supported by the observation that all PcoO6 cells in the 25R2-amended medium had an equal orange color, whereas only a portion of the cells from the control cultures was orange colored.

In summary, we demonstrate that Pluronics alter certain traits of a soil bacterium that are important in its role as a root colonizer and a biocontrol agent. We speculate that the traits of a soil bacterium that are important in its role as a root colonizer and a biocontrol agent. We speculate that the traits of a soil bacterium that are important in its role as a root colonizer and a biocontrol agent. We speculate that the traits of a soil bacterium that are important in its role as a root colonizer and a biocontrol agent. We speculate that the traits of a soil bacterium that are important in its role as a root colonizer and a biocontrol agent.

References


Han SH, Lee SJ, Moon JH et al. (2006) GacS-dependent production of 2R, 3R-butanediol by Pseudomonas chlororaphis O6 is a major determinant for eliciting systemic resistance.

Acknowledgements

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against *Erwinia carotovora* but not against *Pseudomonas syringae* pv. tabaci in tobacco. *Mol Plant Microbe In* **19**: 924–930.


