Original paper

Effect of high growth temperature on *Serratia marcescens*

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**Abstract**

The purpose of this study was to investigate some parameters of *S. marcescens IBB*<sub>Po15</sub> cells adaptation to toxic organic solvents. *S. marcescens IBB*<sub>Po15</sub> cells incubated at 37°C exhibited a slower growth when they were exposed to 5% organic solvents (alkanes, aromatics), as compared with the control cells. *S. marcescens IBB*<sub>Po15</sub> cells grown at 37°C produced protease and the enzyme production decreased in cells exposed to aromatic compounds, as compared with the control cells. Surfactant serrawettin and red pigment prodigiosin were produced by *S. marcescens IBB*<sub>Po15</sub> cells grown at 37°C. Modifications in the pigment and extracellular proteins profile were observed in *S. marcescens IBB*<sub>Po15</sub> cells grown at 37°C during exposure to organic solvents (alkanes, aromatics). Alkane hydroxylase (i.e., *alkB1*) and toluene dioxygenase (i.e., *todM*) catabolic genes, 4′-phosphopantetheinyl transferase (i.e., *pswP*) and serralysin metalloproteinase (i.e., *ser*) genes were detected by polymerase chain reaction in DNA extracted from *S. marcescens IBB*<sub>Po15</sub> control cells grown at 37°C, as well as in cells exposed to organic solvents (excepting *todM* in n-decane exposed cells).

**Keywords** *Serratia marcescens*, solvents, high temperature.

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Introduction

The Gram-negative bacterium *Serratia marcescens* is ubiquitous and persistent in nature and modern artificial environments (S. SUNAGA & al [1]) and it can survive under a wide range of temperatures (5°C-40°C). To respond to the environmental changes, this bacterium has developed elaborate transcriptional regulatory systems which allow their adaptation and proliferation (N.A. STELLA & al [2]). *S. marcescens* is well known for its ability to produce numerous secondary metabolites, including the surfactant serrawettin and the red pigment prodigiosin which are broad-spectrum antibiotics that may aid the bacterium in competition, as well as having different therapeutic potentials (N.A. STELLA & al [2]). *S. marcescens* grown at 30°C produced the red pigment prodigiosin, while at 37°C, this bacterium grow well, but production of this exopolipid was not observed (S. SUNAGA & al [1]; T. MATSUYAMA & al [3]). *S. marcescens* is also well recognized for its ability to produce extracellular metalloproteases with high presence in the stability of solvents and surfactants. Due to these features, these extracellular enzymes are ideal candidates for applications to catalysis in organic solvents systems and other related fields (W. MAO-HUA & al [4]). Solvent-tolerant bacteria isolated from different polluted environments are a relatively novel group of extremophilic microorganisms that combat the destructive effects of high concentrations of toxic organic solvents as a result of several adaptations mechanisms (N. YOGITA & S.B. SARDESSAI [5]).

In this study, we investigated the adaptation mechanisms to toxic organic solvents in *S. marcescens* IBBPola15 cells during exposure to 5% solvents (alkanes: cyclohexane, n-hexane, n-decane, aromatics: toluene, styrene, ethylbenzene), in combination with a high growth temperature (37°C).

Materials and Methods

Overnight culture broth of *S. marcescens* IBBPola15 cells were inoculated on liquid LB-Mg (20 mM MgSO₄) medium (M.M. STANCU & M. GRIFOLL [6]) in the presence of 5% organic solvents (alkanes: cyclohexane, n-hexane, n-decane, aromatics: toluene, styrene, ethylbenzene). Flasks were sealed and incubated for 24 hours at 37°C on a rotary shaker (200 rpm). Two control experiments were set up in parallel, through inoculating of the bacterial cells on liquid LB-Mg medium. Flasks were sealed and incubated for 24 hours at 30°C and 37°C on a rotary shaker (200 rpm).

Cell Growth

Cell growth was determined by measuring the optical density at 660 nm (OD660) using a SPECORD 200 UV-visible spectrophotometer (Analytik Jena, Jena, Germany). Bacterial cultures (20 μl) were spotted on LB-Mg agar for viability assay and on PP agar (protease peptone) for protease assay (C. HENRIETTE & al [7]). Petri plates were incubated for 24 hours at 37°C.

Surfactant Serrawettin and Red Pigment Prodigiosin

Surfactant serrawettin was extracted from the cell-free culture broth with chloroform-methanol (2:1, v/v) (T. MATSUYAMA & al [8]), while red pigment prodigiosin was extracted from the cell pellets with acidified ethanol (K.D. KAMBLE & V.D. HIWARALE [9]). For thin layer chromatography (TLC) analysis, the samples were spotted with a Linomat 5 sample applicator (CAMAG, Muttenz, Switzerland), on a 10x10 cm precoated silica gel 60 TLC aluminum sheets (Merck). The separation was performed using the chloroform-methanol (90:10, v/v) mixture as mobile phase. After development, a densitometric scan at 254 nm in a TLC Scanner 4 (CAMAG) was performed for surfactant and pigment detection and quantification.

Extracellular Proteins

Extracellular proteins were extracted from the cell-free culture broth by ethanol precipitation (Y. SUH & M.J. BENEDIK [10]). Extracted proteins were dissolved in Laemmli buffer and denatured at 96°C, for 10 min. Protein content was determined by measuring optical density at 280 nm (OD280) using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-gelwin system (Biometra, Göttingen, Germany). After electrophoretic separation on 12% polyacrylamide gel and staining with Coomassie brilliant blue (S. SAMBROOK & al [11]) the protein profile was analyzed.

Genomic DNA

Genomic DNA was extracted from the cell pellets with Pure Link genomic kit (Invitrogen, Carlsbad, CA, USA). For polymerase chain reaction (PCR) amplification, 1 μl of DNA extract was added to a final volume of 25 μl reaction mixture, containing: 5×GoTaq flexi buffer, MgCl₂, dNTP mix, specific primers for *alkB1* (ALK3-f and ALK3-r T. KOHNO & al [12]), *todM* (todM-f and todM-r F.J. MÁRQUEZ-ROCHA & al [13]), *pswP* (psrt-f and psrt-r T. TANIKAWA & al [14]) or *ser* (ser-f and ser-r W. MAO-HUA & al [4]) genes, and GoTaq G2 hot start polymerase (Promega, Madison, WI, USA). PCR was performed with a Mastercycler pro S (Eppendorf, Hamburg, Germany). The PCR program consisted in initial denaturation for 10 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C-62°C for 30 sec, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. After electrophoretic separation on 1.5% TBE agarose gel (J. SAMBROOK & al [11]) and staining with fast blast DNA stain (Bio-Rad, Hercules, CA, USA) the PCR products were analyzed.

The sequencing of *pswP* gene PCR products was performed with amplification primers (psrt-f and psrt-r T. TANIKAWA & al [14]) at the CeMIA SA commercial service (Larissa, Greece). The nucleotide sequence of *pswP* gene was compared to that in databases using the BLAST search program (https://blast.ncbi.nlm.nih.gov/BLAST.cgi).
Reagents used during this study were procured from Merck (Darmstadt, Germany), Sigma-Aldrich (Saint-Quentin-Fallavier, France), Promega (Madison, WI, USA), Invitrogen (Carlsbad, CA, USA) or Bio-Rad Laboratories (Hercules, CA, USA). The PCR primers were purchased from Integrated DNA Technologies (Coralville, IA, USA) and Invitrogen (Carlsbad, CA, USA).

Results and Discussion

The solvent-tolerant strain used in this study was *S. marcescens IBBPo15* (KT315653), which has been formerly isolated and characterized by M.M. STANCU [15, 16]. Solvent-tolerant bacteria are commonly well known as ideal candidates for environmental biotechnology applications (N. YOGITA & S.B. SARDESSAI [5]).

**Cell Growth**

As observed in Table 1, *S. marcescens IBBPo15* control cells grew well both at 37°C (OD<sub>660</sub> = 1.13), as well as at 30°C (OD<sub>660</sub> = 1.17). Similar data were previously obtained by T. MATSUYAMA & al [3] for another *S. marcescens* strain cultivated at these two temperatures. *S. marcescens IBBPo15* cells incubated at 37°C exhibited a slower growth when they were exposed to 5% organic solvents (OD<sub>660</sub> = 0.21-0.79), as compared with the control cells at 37°C (OD<sub>660</sub> = 1.13). As expected, alkanes (cyclohexane, *n*-hexane, *n*-decane) were less toxic for this bacterium, compared with aromatics (toluene, styrene, ethylbenzene).

| Assay                          | 30°C Control | Cyclohexane | *n*-Hexane | *n*-Decane | Toluene | Styrene | Ethylbenzene |
|-------------------------------|--------------|-------------|------------|------------|---------|---------|--------------|
| OD<sub>660</sub>              | 1.17 ± 0.05  | 1.13 ± 0.06 | 0.56 ± 0.07 | 0.67 ± 0.06 | 0.79 ± 0.09 | 0.22 ± 0.08 | 0.21 ± 0.06 | 0.26 ± 0.06 |

OD<sub>660</sub> = cell growth was determined by measuring the optical density at 660 nm and the values represent the averages ± standard deviation (SD) of two separate determinations.

These results were confirmed also by the viability and protease assays (Figure 1a, 1b). Confluent cell growth was observed on LB-Mg agar and PP agar for *S. marcescens IBBPo15* control cells and cells exposed to alkanes (cyclohexane, *n*-hexane, *n*-decane) at 37°C, while single colony growth (Figure 1a) or no growth (Figure 1b) was observed for cells exposed to aromatics (toluene, styrene, ethylbenzene). Therefore, the protease production was inhibited when *S. marcescens IBBPo15* cells were exposed to aromatics at 37°C. Characterizations of extracellular proteases from various strains of *S. marcescens* indicate that most strains produce a very similar metalloprotease (N. SALARIZADEH & al [17]).

**Surfactant Serrawettin and Red Pigment Prodiginosin**

When *S. marcescens IBBPo15* control cells and cells exposed to 5% organic solvents were grown at 30°C, both these two secondary metabolites (i.e., surfactant, red pigment) were produced by this bacterium (M.M. STANCU [15]). Therefore, we further investigated by TLC analysis if surfactant serrawettin and red pigment prodiginosin were produced by *S. marcescens IBBPo15* control cells and cells exposed to 5% organic solvents (cyclohexane, *n*-hexane, *n*-decane, toluene, styrene, ethylbenzene) grown at 37°C. According to the literature *S. marcescens* strains produced surfactant serrawettin, and similar to red pigment prodiginosin, their production is thermoregulated (T. MATSUYAMA & al [8]; T. TANIKAWA & al [14]). These secondary metabolites are produced by *S. marcescens* grown at 30°C, but not by the cells grown at 37°C (T. TANIKAWA & al [14]). Prodiginosin production is also dependent on species type, culture conditions and incubation time (T. TANIKAWA & al [14]; A.V. GIRI & al [18]).

*S. marcescens IBBPo15* control cells produced two biosurfactant fractions when they were grown at 37°C (BS<sub>1</sub> with *R*<sub>1</sub> = 0.22, BS<sub>2</sub> with a *R*<sub>2</sub> = 0.27), as well as at 30°C (Figure 2a). The same biosurfactant fractions were observed in the extracts of *S. marcescens IBBPo15* cells exposed to 5% organic solvents at 37°C (BS<sub>1</sub> with *R*<sub>1</sub> = 0.18-0.23, BS<sub>2</sub> = 0.25-0.29). Decrease in biosurfactant production, as well as of their activity was observed in *S. marcescens IBBPo15* control cells and cells exposed to 5% organic solvents at 37°C, as compared with serrawettin production in control cells grown at 30°C.

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**Table 1. Growth of *S. marcescens IBBPo15* cells at 37°C in the presence of 5% organic solvents**

**Figure 1. Growth of *S. marcescens IBBPo15* cells at 37°C in the presence of 5% organic solvents.** a. Viability assay (growth on LB-Mg agar); b. Protease assay (growth on PP agar); control, 30°C (*I*); control, 37°C (*2*); organic solvents, 37°C (*3-8*): cyclohexane (*3*), *n*-hexane (*4*), *n*-decane (*5*), toluene (*6*), styrene (*7*), ethylbenzene (*8*).
Four prodigiosin spots were observed in the extracts of _S. marcescens_ IBBPo15 control cells grown at 37°C (Pg1 with \( R_t = 0.06 \), Pg2 with \( R_t = 0.09 \), Pg3 with \( R_t = 0.13 \), Pg4 with \( R_t = 0.69 \)), and also at 30°C (Figure 2b). Alteration of pigment production was acquired when _S. marcescens_ IBBPo15 cells were exposed to 5% organic solvents at 37°C. However, the pigment production was not totally inhibited in _S. marcescens_ IBBPo15 exposed to 5% organic solvents at 37°C. Re-synthesis of prodigiosin was observed when cultures of _S. marcescens_ IBBPo15 cells exposed to 5% organic solvents at 37°C were incubated at 30°C. Similarly, A.V. GIRI & al [18] observed prodigiosin production for another _S. marcescens_ strain grown at 37°C. Prodigiosin production was observed for _S. marcescens_ cells grown in powdered peanut broth at 37°C, and its production was similar to that obtained in nutrient broth at 30°C (A.V. GIRI & al [18]).

**Figure 2.** Surfactant serrawettin and red pigment prodigiosin profile of _S. marcescens_ IBBPo15 cells grown at 37°C in the presence of 5% organic solvents. a. TLC analysis of surfactant serrawettin; b. TLC analysis of red pigment prodigiosin; control, 30°C (I); control, 37°C (2); organic solvents, 37°C (3-8): cyclohexane (3), n-hexane (4), n-decane (5), toluene (6), styrene (7), ethylbenzene (8); biosurfactants (BS1, BS2); prodigiosin (Pg1-Pg4).

**Extracellular Proteins**

Proteins with estimated molecular weights below 75 kDa (including 50 kDa protein) were detected by SDS-PAGE studies (Figure 3) in the extracts of _S. marcescens_ IBBPo15 control cells and cells exposed to 5% organic solvents at 37°C, and also in the control cells grown at 30°C. Similar data were obtained by M.M. STANCU [16] when _S. marcescens_ IBBPo15 cells grown at 30°C where exposed to 5% organic solvents. The production of the 50 kDa protein by _S. marcescens_ IBBPo15 cells was affected by organic solvents exposure of the cells (M.M. STANCU [16]). Modifications in the extracellular proteins profile (including 50, 40, 20, 18 and 16 kDa protein) were observed in _S. marcescens_ IBBPo15 cells grown at 37°C and exposed to 5% organic solvents, as compared with proteins profile of control cells grown at 37°C and 30°C. The _S. marcescens_ metalloprotease (50 kDa protein) is a member of the serralysin family of proteolytic enzymes which is important for different biomedical applications (N. SALARIZADEH & al [17]).

**Figure 3.** Proteins profile of _S. marcescens_ IBBPo15 cells grown at 37°C in the presence of 5% organic solvents. SDS-PAGE analysis of extracellular proteins; control, 30°C (I); control, 37°C (2); organic solvents, 37°C (3-8): cyclohexane (3), n-hexane (4), n-decane (5), toluene (6), styrene (7), ethylbenzene (8); broad range protein molecular weight marker, Promega (M).

**Genomic DNA**

Genomic DNA extracted from _S. marcescens_ IBBPo15 control cells and from cells exposed to 5% organic solvents at 37°C was used as template for PCR amplification of known alkane hydroxylase (i.e., _alkB1_) and toluene dioxygenase (i.e., _todM_) catabolic genes, 4'-phosphopantetheinytransferase (i.e., _pswP_) and serralysin metalloproteinase (i.e., _ser_) genes (Figure 4a-4d). S.M. STANCU [15] showed formerly that _alkB1_, _todM_, _pswP_ and _ser_ genes were detected in DNA extracted from _S. marcescens_ IBBPo15 control cells grown at 30°C, as well as in cells exposed to 5% organic solvents. _S. marcescens_ IBBPo15 _pswP_ gene exhibited nucleotide sequence similarity levels of 99% with respect to the sequences of the
4'-phosphopantetheinyl transferase gene from *S. marcescens* (AB163428.1). As expected, *alkB1* (330 bp fragment), *todM* (560 bp), *pswP* (560 bp) and *ser* (1515 bp) genes were detected also in DNA extracted from *S. marcescens* IBB<sub>Po15</sub> control cells grown at 37°C (Figure 4a-4d), as well as in cells exposed to 5% organic solvents at 37°C (excepting *todM* in n-decane exposed cells). Surprisingly, in DNA extracted from *S. marcescens* IBB<sub>Po15</sub> cells grown at 37°C and exposed to n-decane, *todM* gene (560 bp) was not detected. The amplification of *todM* gene in n-decane exposed gene was in barely quantity, as compared with their amplification in control cells grown at 37°C.

**Figure 4.** Detection of *alkB1*, *todM*, *pswP* and *ser* genes in DNA extracted from *S. marcescens* IBB<sub>Po15</sub> cells grown at 37°C in the presence of 5% organic solvents. a. PCR of *alkB1* gene (expected fragment size 330 bp); b. PCR of *todM* gene (expected fragment size 560 bp); c. PCR of *pswP* gene (expected fragment size 560 bp); d. PCR of *ser* gene (expected fragment size 1515 bp); control, 30°C (1); control, 37°C (2); organic solvents, 37°C (3-8): cyclohexane (3), n-hexane (4), n-decane (5), toluene (6), styrene (7), ethylbenzene (8); 1 kb DNA ladder, Promega (M).

**Conclusions**

*Serratia marcescens* is a Gram-negative entero-bacterium which is omnipresent in many environmental niches, including in sites polluted with petroleum products in a large range of temperatures. *S. marcescens* IBB<sub>Po15</sub> cells incubated at 37°C exhibited a slower growth when they were exposed to 5% organic solvents (alkanes, aromatics), as compared with the control cells. *S. marcescens* IBB<sub>Po15</sub> cells grown at 37°C produced protease and the enzyme production decreased in cells exposed to aromatic compounds. Surfactant serrawettin and red pigment prodigiosin were produced by *S. marcescens* IBB<sub>Po15</sub> cells, and alteration of pigment profile was acquired in cells grown at 37°C and exposed to organic solvents. Modifications in the extracellular proteins profile were observed in *S. marcescens* IBB<sub>Po15</sub> cells grown at 37°C during exposure to organic solvents. In DNA extracted from *S. marcescens* IBB<sub>Po15</sub> cells grown at 37°C and exposed to organic solvents were detected *alkB1*, *todM*, *pswP* and *ser* genes (excepting *todM* in n-decane exposed cells).

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