

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_{Po15} cells adaptation to toxic organic solvents. *S. marcescens* IBB_{Po15} 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_{Po15} 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_{Po15} cells grown at 37°C. Modifications in the pigment and extracellular proteins profile were observed in *S. marcescens* IBB_{Po15} 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 serralyisin metalloproteinase (i.e., *ser*) genes were detected by polymerase chain reaction in DNA extracted from *S. marcescens* IBB_{Po15} 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

1. 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 exolipid 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 stability in the presence 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* IBB_{Po15} 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).

2. Materials and Methods

Overnight culture broths of *S. marcescens* IBB_{Po15} 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 (proteose 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 broths 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 10×10 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 broths 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 Minigel-twin system (Biometra, Göttinger, 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).

3. Results and Discussion

The solvent-tolerant strain used in this study was *S. marcescens* IBB_{P015} (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* IBB_{P015} control cells grew well both at 37°C (OD₆₆₀ = 1.13), as well as at 30°C (OD₆₆₀ = 1.17). Similar data were previously obtained by T. MATSUYAMA & al. [3] for another *S. marcescens* strain cultivated at these two temperatures. *S. marcescens* IBB_{P015} cells incubated at 37°C exhibited a slower growth when they were exposed to 5% organic solvents (OD₆₆₀ = 0.21-0.79), as compared with the control cells at 37°C (OD₆₆₀ = 1.13). As expected, alkanes (cyclohexane, *n*-hexane, *n*-decane) were less toxic for this bacterium, compared with aromatics (toluene, styrene, ethylbenzene).

Table 1. Growth of *S. marcescens* IBB_{P015} cells at 37°C in the presence of 5% organic solvents

Assay	Growth temperature							
	30°C	37°C						
	Control	Control	Cyclohexane	<i>n</i> -Hexane	<i>n</i> -Decane	Toluene	Styrene	Ethylbenzene
OD ₆₆₀	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

OD₆₆₀ = 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* IBB_{P015} 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* IBB_{P015} 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]).

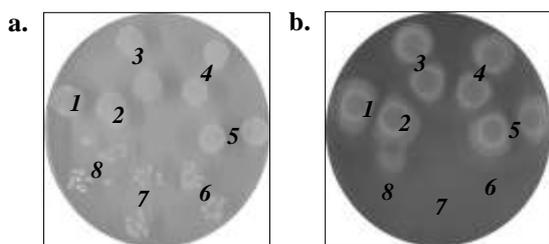


Figure 1. Growth of *S. marcescens* IBB_{P015} cells at 37°C in the presence of 5% organic solvents. **a.** Viability assay (growth on LB-Mg agar); **b.** Protease assay (growth on PP agar); control, 30°C (1); control, 37°C (2); organic solvents, 37°C (3-8): cyclohexane (3), *n*-hexane (4), *n*-decane (5), toluene (6), styrene (7), ethylbenzene (8).

Surfactant Serrawettin and Red Pigment Prodigiosin.

When *S. marcescens* IBB_{P015} 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 prodigiosin were produced by *S. marcescens* IBB_{P015} 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 prodigiosin, 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]). Prodigiosin production is also dependent on species type, culture conditions and incubation time (T. TANIKAWA & al. [14]; A.V. GIRI & al. [18]).

S. marcescens IBB_{P015} control cells produced two biosurfactant fractions when they were grown at 37°C (BS₁ with $R_f = 0.22$, BS₂ with a $R_f = 0.27$), as well as at 30°C (Figure 2a). The same biosurfactant fractions were observed in the extracts of *S. marcescens* IBB_{P015} cells exposed to 5% organic solvents at 37°C (BS₁ with $R_f = 0.18-0.23$, BS₂ = 0.25-0.29). Decrease in biosurfactant production, as well as of their activity was observed in *S. marcescens* IBB_{P015} control cells and cells exposed to 5% organic solvents at 37°C, as compared with serrawettin production in control cells grown at 30°C.

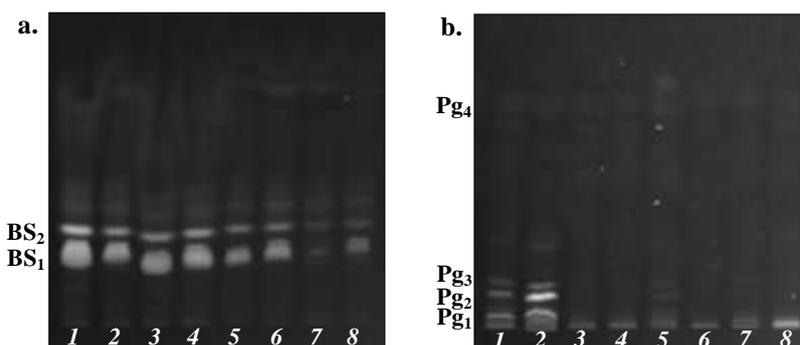


Figure 2. Surfactant serrawettin and red pigment prodigiosin profile of *S. marcescens* IBB_{P015} cells grown at 37°C in the presence of 5% organic solvents. **a.** TLC analysis of surfactant serrawettin; **b.** TLC analysis of red pigment prodigiosin; control, 30°C (1); control, 37°C (2); organic solvents, 37°C (3-8): cyclohexane (3), *n*-hexane (4), *n*-decane (5), toluene (6), styrene (7), ethylbenzene (8); biosurfactants (BS₁, BS₂); prodigiosin (Pg₁-Pg₄).

Four prodigiosin spots were observed in the extracts of *S. marcescens* IBB_{P015} control cells grown at 37°C (Pg₁ with $R_f = 0.06$, Pg₂ with $R_f = 0.09$, Pg₃ with $R_f = 0.13$, Pg₄ with $R_f = 0.69$), and also at 30°C (Figure 2b). Alteration of pigment production was acquired when *S. marcescens* IBB_{P015} cells were exposed to 5% organic solvents at 37°C. However, the pigment production was not totally inhibited in *S. marcescens* IBB_{P015} exposed to 5% organic

solvents at 37°C. Re-synthesis of prodigiosin was observed when cultures of *S. marcescens* IBB_{P015} 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]).

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* IBB_{P015} 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* IBB_{P015} cells grown at 30°C were exposed to 5% organic solvents. The production of the 50 kDa protein by *S. marcescens* IBB_{P015} 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* IBB_{P015} 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 serralyisin family of proteolytic enzymes which is important for different biomedical applications (N. SALARIZADEH & al. [17]).

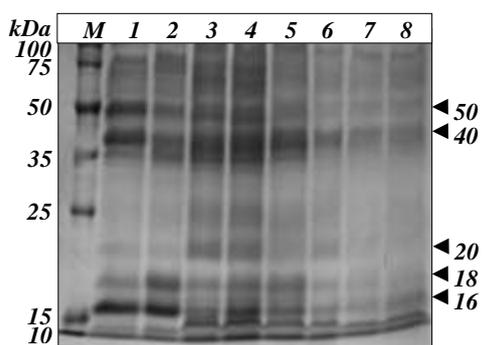


Figure 3. Proteins profile of *S. marcescens* IBB_{P015} cells grown at 37°C in the presence of 5% organic solvents. SDS-PAGE analysis of extracellular proteins; control, 30°C (1); control, 37°C (2); organic solvents, 37°C (3-8): cyclohexane (3), *n*-hexane (4), *n*-decane (5), toluene (6), styrene (7), ethylbenzene (8); broad range protein molecular weight marker, Promega (M).

Genomic DNA.

Genomic DNA extracted from *S. marcescens* IBB_{P015} 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'-phosphopantetheinyl transferase (i.e., *pswP*) and serralyisin 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* IBB_{P015} control cells grown at 30°C, as well as in cells exposed to 5% organic solvents. *S. marcescens* IBB_{P015} *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_{P015} 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_{P015} 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.

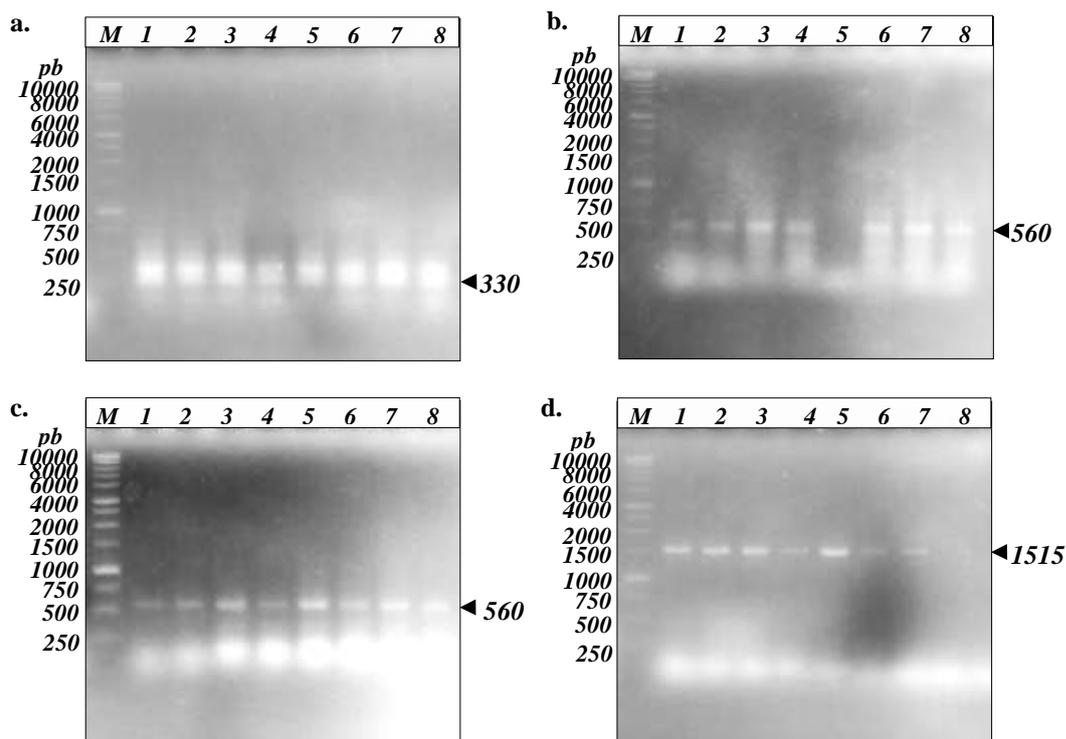


Figure 4. Detection of *alkB1*, *todM*, *pswP* and *ser* genes in DNA extracted from *S. marcescens* IBB_{P015} cells grown at 37°C in the presence of 5% organic solvents. **a.** PCR of *alkB1* gene (expected fragment size 330 bp); **b.** PCR of *todM* gene (expected fragment size 560 bp); **c.** PCR of *pswP* gene (expected fragment size 560 bp); **d.** PCR of *ser* gene (expected fragment size 1515 bp); control, 30°C (**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**); 1 kb DNA ladder, Promega (**M**).

4. Conclusions

Serratia marcescens is a Gram-negative enterobacterium which is omnipresent in many environmental niches, including in sites polluted with petroleum products in a large range of temperatures. *S. marcescens* IBB_{P015} 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_{P015} 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_{P015} 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_{P015} cells grown at 37°C during exposure to organic solvents. In DNA extracted from *S. marcescens* IBB_{P015} 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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