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Highly Solvent Tolerance in *Serratia marcescens* IBB_{Po15}

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ABSTRACT

The aim of this study was to investigate the solvent tolerance mechanisms in Serratia marcescens strain IBB_{Po15} (KT315653). Serratia marcescens IBB_{Po15} exhibited remarkable solvent-tolerance, being able to survive in the presence of high concentrations (above 40%) of toxic organic solvents, such as cyclohexane, n-hexane, n-decane, toluene, styrene, and ethylbenzene. S. marcescens IBB_{Po15} produced extracellular protease and the enzyme production decreased in cells exposed to 5% cyclohexane, n-hexane, toluene, styrene, and ethylbenzene, as compared with the control and n-decane exposed cells. S. marcescens IBB_{Po15} cells produced carotenoid pigments and alteration of pigments profile (i.e., phytoene, lycopene) were observed in cells exposed to 5% cyclohexane, n-hexane, n-decane, toluene, styrene, and ethylbenzene. The exposure of S. marcescens IBB_{Po15} cells to 5% cyclohexane, n-hexane, n-decane, toluene, styrene, ethylbenzene induced also changes in the intracellular (e.g., 50 kDa protein) and extracellular (e.g., 39, 41, 43, 53, 110 kDa proteins) proteins profile. Significant RAPD, ARDRA, rep-PCR and PCR pattern modifications were not observed in DNA extracted from S. marcescens IBB_{Po15} cells exposed to 5% cyclohexane, n-hexane, n-decane, toluene, styrene, and ethylbenzene. Though only HAE1 and acrAB genes were detected in the genome of S. marcescens IBB_{Po15} cells, the unspecific amplification of other fragments being observed also when the primers for ompF and recA genes were used.

Key words: Serratia marcescens; solvents; tolerance; mechanisms

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INTRODUCTION

Serratia marcescens is a Gram-negative bacillus, classified as a member of *Enterobacteriaceae* family, which is found in different ecological niches, including soil, water, air, plants and animals. Among enteric bacteria, *S. marcescens* is unique, because it secretes extracellular enzymes (e.g., chitinase, DNase, lipase, protease), produces red pigment prodigiosin and surfactant serrawettin used in the surfaces colonization [1]. Carotenoid pigments (e.g., β-carotene) are produced also in *S. marcescens* [2], and the pigment biosynthesis acts as a protective mechanism in unfavorable conditions [1]. Bacterial strains living in the polluted environments must cope with the toxic compounds (e.g., organic solvents) which result from humans activity. Surface bacterial structures, cell wall and cytoplasmic membrane, surround each bacterial cell and create selective barriers between the cell interior and the outside of the cell [3]. They are the first site of contact between the cell and toxic compounds which are able to penetrate into cytoplasmic membrane and affect the membrane functions [3,4]. Therefore, bacteria developed adaptation mechanisms to counteract the damage originated from toxic compounds and to prevent their accumulation inside of the cell [3]. Membrane transporters, such as porins or efflux pumps, are main filters regulating the internal accumulation of toxic compounds [4]. Porins OmpF (with molecular weight of 41 kDa) and OmpC (40 kDa) which exist in outer membrane of *S. marcescens* cells are two non-specific protein channels that serve to take in nutrients and toxic compounds, and export waste products [5,6]. Furthermore, the AcrAB-TolC efflux system is a tripartite complex and is a member of the resistance-nodulation-division (RND) family which is considered the major efflux system in members of *Enterobacteriaceae* family [7] responsible for tolerance to toxic compounds, including organic solvents [8]. The drug:proton antiporter AcrB (110 kDa), which is located in the inner membrane, captures its substrates and transports them into the external medium via the outer membrane channel TolC (53 kDa) [8,9]. The cooperation between AcrB and TolC is mediated by the periplasmic protein AcrA (43 kDa) [9] and the activity of the AcrAB efflux pump is under the control of stress response genes [8]. The role of TolC in enterobacteria physiology is very broad and inactivation of *tolC* gene affects the cell adaptation to adverse environments [10]. Toxic organic compounds that enter inside of the cell cause sometimes DNA damage which activates the SOS response in Gram-negative bacteria. Induction of the SOS response involves over forty independent SOS genes, most of them encoding proteins involved in protection, repair, replication, mutagenesis and DNA metabolism. When the DNA is damaged, RecA protein (39 kDa) binds to the single stranded DNA to form a nucleoprotein and acquires a co-protease activity which leads to the self-cleavage of LexA protein from the SOS box [11].

In order to use of the solvent-tolerant bacteria in bioremediation of polluted environments, much research has been performed to investigate the mechanisms of solvent tolerance in these bacteria. However, the tolerance mechanisms of many bacteria are still not very obvious [12]. In this study, we investigated the solvent tolerance mechanisms in *Serratia marcescens* IBB_{Po15} cells by following the changes in the membrane permeability, carotenoid pigments profile, protein profile, and DNA patterns.

MATERIALS AND METHODS

Bacterial Strain

The strain used in the present study was *Serratia marcescens* IBB_{Po15} (KT315653), which has been previously isolated by us from an oily sludge sample [13].

Solvent Tolerance

For further characterization of *S. marcescens* IBB_{Po15}, the viability in the presence of 1-100% organic solvents was investigated. Bacterial cells were cultivated on liquid LB medium [13] and incubated at 30°C on a rotary shaker (200 rpm) until they reached a turbidity of 0.400 (OD_{660nm}). Then, 1-100% organic solvents (alkanes: cyclohexane, *n*-hexane, *n*-decane; aromatics: toluene, styrene, ethylbenzene) with logarithm of the partition coefficient of the solvent in octanol-water mixture ($\log P_{OW}$) between 2.64 and 5.98 were supplied to the culture broths. Tubes were sealed and incubated for 1 hour at 30°C on a rotary shaker (200 rpm). Then, the cell viability in the presence of organic solvents was determined. Bacterial cultures after organic solvents exposure (20 µl) were spotted on LB agar. Petri plates were incubated for 24 hours at 30°C. Here, as elsewhere in this work the assays were done in duplicate.

Solvents used during this study were of reagent grade and purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Plate overlay assay was also used to investigate solvent tolerance in *S. marcescens* IBB_{Po15}. Bacterial cultures with a turbidity of 0.400 (20 µl) were spotted on LB agar. Petri plates were incubated for 30 min at 30°C. Then, organic solvents were poured on top of the LB agar plate surface to a depth of 5 mm. After 1 hour the organic solvents were pipetted off, and the Petri plates were incubated for 24 hours at 30°C.

Bacterial cells were cultivated on liquid LB medium and incubated at 30°C on a rotary shaker (200 rpm) until they reached a turbidity of 0.400 (OD_{660nm}). Then, 5% organic solvents were supplied to the culture broths. Flasks were sealed and incubated for 24 hours at 30°C on a rotary shaker (200 rpm).

Cell Membrane Permeability

Cell membrane permeability was determined using the method indicated by Gaur and Khare [14]. The absorbance of cell-free culture broths was measured at 260 nm using a SPECORD 200 UV-visible spectrophotometer (Analytik Jena, Jena, Germany).

Carotenoid Pigments

Carotenoids were extracted from the cell pellets with acetone [15]. 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 detection and quantification of pigments. The same plate was put into a jar saturated with iodine vapors to detect lipids in the pigments extracts [16]. The plate was also treated with orcinol in H_2SO_4 to detect sugars in the pigments extracts [16]. UV/visible scanning spectra of the pigments extracts were recorded between 200 and 800 nm using a SPECORD 200 UV-visible spectrophotometer (Analytik Jena).

Proteins

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, **extracellular proteins** were extracted from the cell-free culture broths by ethanol precipitation and **intracellular proteins** were extracted from cell pellets by sonication [17]. Extracted proteins were dissolved in Laemmli buffer and denaturated at 96°C, for 10 min. Protein content was determined by measuring optical density (OD) at 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). 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 [18] the protein profile was analyzed.

Molecular Characterization

Genomic DNA was extracted from the cell pellets with Pure Link genomic kit (Invitrogen, Carlsbad, CA, USA). The PCR primers used in this study were purchased from Biosearch Technologies (Novato, CA, USA), Integrated DNA Technologies (Coralville, IA, USA) and Invitrogen (Carlsbad, CA, USA).

Random Amplification Of DNA Fragments (RAPD)

For RAPD, 1 µl of DNA extract was added to a final volume of 25 µl reaction mixture, containing: 5×GoTaq flexi buffer, $MgCl_2$, dNTP mix, primers (AP5, [19]; AP12, [20]), 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 45 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. After electrophoretic separation on 2% TBE agarose gel [18] and staining with fast blast DNA stain (Bio-Rad, Hercules, CA, USA) the PCR products were analyzed.

Repetitive Sequence-Based PCR (rep-PCR)

For rep-PCR amplification, 1 µl of DNA extract was added to a final volume of 25 µl reaction mixture, containing: 5×GoTaq flexi buffer, $MgCl_2$, dNTP mix, primers (REP 1R-Dt and REP 2-Dt, BOXA 1R, $(GTG)_5$, [21]), and GoTaq G2 hot start polymerase (Promega). 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 40°C or 50°C for 1

min, extension at 65°C for 8 min, and a final extension at 65°C for 16 min. After electrophoretic separation on 1.5% TBE agarose gel [18] and staining with fast blast DNA stain (Bio-Rad) the rep-PCR products were analyzed.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

For PCR amplification of 16S ribosomal RNA (16S rRNA) gene, 2 µl of DNA extract were added to a final volume of 50 µl reaction mixture, containing: 5×GoTaq flexi buffer, $MgCl_2$, dNTP mix, universal bacterial primers (27f and 1492r, [22]), and GoTaq G2 hot start polymerase (Promega). 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 55°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 [18] and staining with fast blast DNA stain (Bio-Rad) the PCR products were analyzed. PCR products were digested (at 37°C for 1-3 h) with *Alu*I, *Eco*RI, *Hae*III and *Hha*I restriction endonucleases (Promega), and the resultant restriction fragments were analyzed by electrophoresis on 2.5% agarose gel [18] and staining with fast blast DNA stain (Bio-Rad).

Polymerase Chain Reaction (PCR)

For PCR amplification, 1 µl of DNA extract was added to a final volume of 25 µl reaction mixture, containing: 5×GoTaq flexi buffer, $MgCl_2$, dNTP mix, specific primers for *ompF*, *HAE1*, *acrAB* transporter genes (ompF-f and ompF-r, [6]; A24f2 and A577r2, [23]; acrA-f and acrB-r, [7]), *recA* gene (recA-f and recA-r, [24]), and GoTaq G2 hot start polymerase (Promega). 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 or 55°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 [18] and staining with fast blast DNA stain (Bio-Rad) the PCR products were analyzed.

RESULTS AND DISCUSSION

S. marcescens strain IBBPo15 (16S rRNA gene, GenBank KT315653) has been previously isolated by us from an oily sludge sample [13].

During the exposure of *S. marcescens* IBB_{Po15} cells to 5% organic solvents, *n*-decane was less toxic for this bacterium, compared with cyclohexane, *n*-hexane, toluene, styrene, and ethylbenzene [13]. *S. marcescens* IBB_{Po15} cells which possess *pswP* (4'-phosphopantetheinyl transferase) gene produced some secondary metabolites, such as surfactant serrawettin and red pigment prodigiosin [13]. The detection of *pswP* gene in *S. marcescens* IBB_{Po15} cells was not surprising since Tanikawa et al. [25] showed formerly that the *pswP* gene is essential for the synthesis of serrawettin and prodigiosin in *S. marcescens*.

Proteases production by *S. marcescens* IBB_{Po15} control cells and cells exposed to 5% organic solvents was further investigated. Bacterial cultures (20 µl) after organic solvents exposure were spotted on proteose peptone agar [26]). Petri plates were incubated for 24 hours at 30°C. Protease activity was lower (below 75%) after cyclohexane, *n*-hexane, toluene, styrene, and ethylbenzene exposure of *S. marcescens* IBB_{Po15} cells, compared with the control and *n*-decane exposed cells (100%). Henriette et al. [26] reported that the production of protease by *Serratia marescens* 532 S was influenced by nutritional factors which acted as inducers or repressors.

Solvent Tolerance

For further characterization of *S. marcescens* IBB_{Po15}, the viability in the presence of 1-100% organic solvents was investigated. As could be observed in Figure 1, *S. marcescens* IBB_{Po15} cells were able to survive (confluent cell growth) in the presence of 80% cyclohexane, 100% *n*-decane and *n*-hexane, 60% toluene, styrene, and 40% ethylbenzene. These results were confirmed by the INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) method. No growth was observed when *S. marcescens* IBB_{Po15} cells were exposed to 80% toluene, styrene, and 60% ethylbenzene. In plate overlay assay, *S. marcescens* IBB_{Po15} was able to survive (confluent cell growth) in 100% *n*-decane, *n*-hexane, cyclohexane, while 100% toluene, styrene and ethylbenzene totally inhibited cell growth. The absence of the bacterial cells growth indicated that organic solvents with aromatic structure (i.e., toluene, styrene, ethylbenzene) were more toxic for *S. marcescens* IBB_{Po15} cells, compared with alkanes (i.e., cyclohexane, *n*-hexane, *n*-decane). According to the literature [27], the solvents with log P_{OW} values less than 4.0 are considered to be extremely toxic. Every bacterium has limiting log P_{OW} values below which it is unable to grow and

this intrinsic tolerance level is determined by genetic and environmental factors [27]. Therefore, the bacteria which are able to grow on and utilize saturated concentrations of organic solvents can be used in the removal of such pollutants [27] and biphasic catalysis [12].

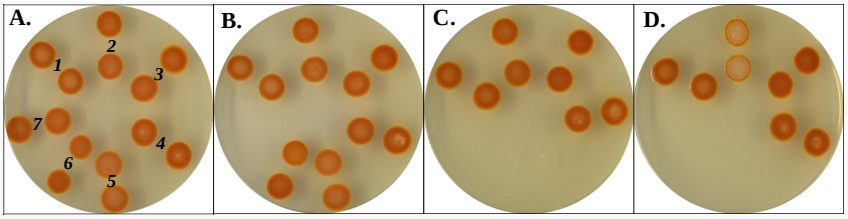

Figure 1 The viability of *S. marcescens* IBB_{Po15} cells in the presence of 40% (**A**), 60% (**B**), 80% (**C**) and 100% (**D**) organic solvents. Control (***1***), cyclohexane (***2***), *n*-hexane (***3***), *n*-decane (***4***), toluene (***5***), styrene (***6***), ethylbenzene (***7***). Confluent cell growth or no growth was observed.

Cell Membrane Permeability

Like in other Gram-negative bacteria, the envelope of *S. marcescens* is an important barrier that protects the cell against toxic compounds [4]. However, toxic organic solvents are able to penetrate into cytoplasmic membrane resulting in swelling of the membrane and increase of membrane fluidity which leads to the loss of membrane functionality and to the damage of bacterial cell. The leakage of DNA macromolecules was previously described [14] for the bacterial cells grown in the presence of toxic organic solvents. Bacterial cells try to undertake proper responses to reduce the disruptive effect of organic compounds by readjustment of membrane fluidity [3]. The release of nucleic acids from *S. marcescens* IBB_{Po15} control cells and cells exposed to 5% organic solvents was investigated by measuring the absorbance of cell-free culture broths at 260 nm. The release of nucleic acids from *S. marcescens* IBB_{Po15} (Table 1) was lower (OD = 0.022, 0.027) in the case of the control and *n*-decane exposed cells, compared with the release of nucleic acids from cells exposed to cyclohexane, *n*-hexane, styrene, toluene and ethylbenzene (OD = 0.059-0.082). Similar data were previously obtained by Stancu [28] for another Gram-negative bacterium, *Shewanella putrefaciens* IBB_{Po6} exposed to 5% organic solvents.

Table 1 Permeability of *S. marcescens* IBB_{Po15} cells after 5% organic solvents exposure

Assay	Organic solvents (log P_{OW})						
	Control	Cyclohexane (3.35)	*n*-Hexane (3.86)	*n*-Decane (5.98)	Toluene (2.64)	Styrene (2.86)	Ethylbenzene (3.17)
Cell permeability	0.022	0.067	0.059	0.027	0.073	0.082	0.061

Cell permeability, the absorbance of cell-free culture broths was measured at 260 nm. Logarithm of the partition coefficient of the solvent in octanol-water mixture (log P_{OW}).

Carotenoids

Pigments derived from natural sources (e.g., bacteria) have emerged as an important alternative to synthetic food colorants which have different harmful effects on human health. *S. marcescens* strains produced carotenoids pigments, such as β-carotene which is a commonly used food colorant with antioxidant and pro-vitamin activities. Carotenoids can inhibit various types of cancer and it enhances the immune response [2]. We further investigated carotenoid pigments production in *S. marcescens* IBB_{Po15} control cells and cells exposed to 5% organic solvents. The UV/visible absorption scanning spectra of the pigments extracts of *S. marcescens* IBB_{Po15} control cells showed absorption maxima at 335 nm. Carotenoid pigments extracted from cells exposed to *n*-decane, toluene, styrene and ethylbenzene showed absorption maxima at 335 nm, while cyclohexane and *n*-hexane exposed cells showed absorption maxima at 340 nm. According to the literature [29], some bacteria can accumulate carotenoid pigments as part of their responses to different

environmental stresses, aiding in this way their survival in these habitats.

Six carotenoid pigments were detected by TLC analysis (at 254 nm) in the extracts of *S. marcescens* IBB_{Po15} control cells: phytoene (Pe) with R_f (retardation factor) 0.04, lycopene (Ly) with R_f 0.07, 4-keto-γ-carotene (KγC) with R_f 0.16, chlorobactene (CB) with R_f 0.20, γ-carotene (γC) with R_f 0.50, and β-carotene (βC) with R_f 0.72 (Fig. 2A, 2B). The same carotenoids were detected in the extracts of *S. marcescens* IBB_{Po15} cells exposed to 5% organic solvents: Pe with R_f 0.03-0.06, Ly with R_f 0.07-0.09, KγC with R_f 0.15-0.16, CB with R_f 0.20, γC with R_f 0.50, and βC with R_f 0.70-0.74. An elevated level of Pe and especially Ly were detected in the extracts of *S. marcescens* IBB_{Po15} control cells and cells exposed to 5% organic solvents, as compared with other carotenoid pigments (i.e., KγC, CB, γC, βC) which were detected only in barely quantities. All the carotenoid pigments were visible as light brown spots (at 500 nm) when the plate was kept into a jar saturated with iodine vapors (Fig. 2C). Pe and Ly were visible (at 366 nm) as light blue spots (Fig. 2D) on the plate treated with orcinol in H_2SO_4; KγC, CB, γC, and βC were detected in barely quantities on orcinol treated plate. Treatment of the TLC plate with iodine vapors or with orcinol in H_2SO_4 could be also used for detection and quantification of the carotenoid pigments. TLC analysis showed the existence of some differences between carotenoid pigments profile of *S. marcescens* IBB_{Po15} control cells and that of cells exposed to 5% organic solvents (i.e., Pe, Ly). Therefore, modifications of these secondary metabolites profile were observed when *S. marcescens* IBB_{Po15} cells were exposed to 5% organic solvents.

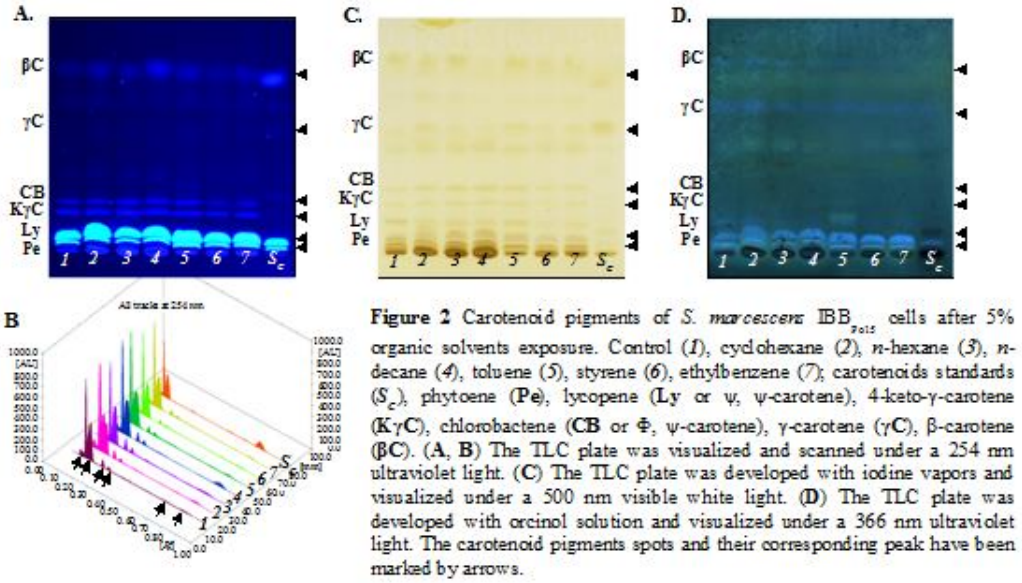

Figure 2 Carotenoid pigments of *S. marcescens* IBB_{Po15} cells after 5% organic solvents exposure. Control (*1*), cyclohexane (*2*), *n*-hexane (*3*), *n*-decane (*4*), toluene (*5*), styrene (*6*), ethylbenzene (*7*); carotenoids standards (S_c), phytoene (**Pe**), lycopene (**Ly** or ψ, ψ-carotene), 4-keto-γ-carotene (**KγC**), chlorobactene (**CB** or Φ, ψ-carotene), γ-carotene (**γC**), β-carotene (**βC**). (**A**, **B**) The TLC plate was visualized and scanned under a 254 nm ultraviolet light. (**C**) The TLC plate was developed with iodine vapors and visualized under a 500 nm visible white light. (**D**) The TLC plate was developed with orcinol solution and visualized under a 366 nm ultraviolet light. The carotenoid pigments spots and their corresponding peak have been marked by arrows.

Proteins

The extracellular and intracellular proteins extracted from *S. marcescens* IBB_{Po15} control cells and from cells exposed to 5% organic solvents were analyzed by SDS-PAGE. The exposure of *S. marcescens* IBB_{Po15} cells to toxic organic solvents had different effects on the proteins profile (Fig. 3A, 3B).

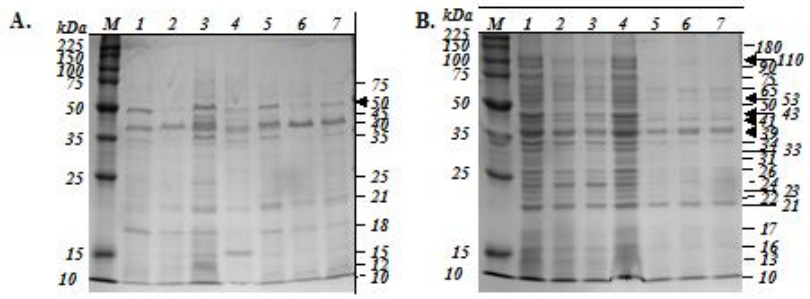

Figure 3 Extracellular (**A**) and intracellular (**B**) proteins profile of *S. marcescens* IBB_{Po15} cells after 5% organic solvents exposure. Control (*1*), cyclohexane (*2*), *n*-hexane (*3*), *n*-decane (*4*), toluene (*5*), styrene (*6*), ethylbenzene (*7*); broad range protein molecular weight marker, Promega (*M*). The expected position for 50 kDa metalloproteinase, 110 kDa AcrB, 43 kDa AcrA, 41 kDa OmpF, and 39 kDa RecA has been marked by arrows.

Proteins with estimated molecular weights between 10 and 75 kDa were detected in the **extracellular extracts** from *S. marcescens* IBB_{Po15} control cells, while the induction of proteins with molecular weights between 12 and 75 kDa was observed in the extracts from *S. marcescens* IBB_{Po15} cells exposed to *n*-hexane (Fig. 3A). Proteins with molecular weights between 15 and 40 kDa were induced in *n*-decane, toluene, styrene and ethylbenzene exposed cells. No significant differences were observed between proteins profile of control cells and that of the cells exposed to cyclohexane (except for a 50 kDa protein). The concentration of a protein of about 50 kDa increased in *n*-hexane exposed cells, while in cyclohexane, *n*-decane, toluene, styrene and ethylbenzene exposed cells its concentration decreased. The production of the 50 kDa protein by *S. marcescens* IBB_{Po15} cells was affected by organic solvents (except *n*-decane) exposure of the cells. We presume that this protein with molecular weight of about 50 kDa correspond to an extracellular metalloproteinase (known also as serratiopeptidase, serratia peptidase, serralysin metalloproteinase) of the serralysin superfamily described formerly for other *S. marcescens* strains. The primary function of serratiopeptidases is nutrient uptake, but they are also involved in pathogenesis of *S. marcescens* [30,31].

Proteins with estimated molecular weights between 10 and 180 kDa were detected in the **intracellular extracts** from *S. marcescens* IBB_{Po15} control cells. We consider that the bands with molecular weight of about 39 correspond to RecA protein and 41 kDa correspond to OmpF proteins. Furthermore, the bands with molecular weight of about 43, 53, and 110 kDa correspond to AcrA, TolC, and AcrB proteins, correspondingly. We observed (Fig. 3B), the repression of proteins with molecular weights between 13 and 110 kDa in the extracts from *S. marcescens* IBB_{Po15} cells exposed to cyclohexane, *n*-hexane, toluene, styrene and ethylbenzene, as compared with proteins profile of control cells. No significant changes were observed in the proteins profile of the cells exposed to *n*-decane which seemed to be less toxic for this bacterium. Toxic organic solvents usually affect the cell proteome [3]. Because under nutrient limitation conditions damaged proteins cannot be replaced with the newly synthesized, the proteome repair is required to maintain cell vitality. Three major mechanisms operate in bacteria after a proteome damage induced by solvent exposure (i.e., chaperones synthesis, activation of enzymatic repair systems, proteolysis of abnormal proteins which cannot be repaired) [3].

Molecular Characterization

Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers [32]. Several molecular biology techniques have been further used to study solvent tolerance mechanisms in *S. marcescens* IBB_{Po15}.

Random Amplification Of DNA Fragments (RAPD)

Genomic DNA extracted from *S. marcescens* IBB_{Po15} control cells and from cells exposed to 5% organic solvents was used as template for RAPD, using AP5 and AP12 primers (Fig. 4A, 4B). The annealing temperature was 36°C for both these two primers. Fragments with sizes ranging from 350 to 2000 bp were detected when AP5 and AP12 primers were used. Considerable modifications of the RAPD patterns were not observed when *S. marcescens* IBB_{Po15} cells were exposed to

cyclohexane, *n*-hexane, *n*-decane, toluene, styrene, and ethylbenzene.

Repetitive Sequence-Based PCR (rep-PCR)

Genomic DNA extracted from *S. marcescens* IBB_{Po15} control cells and from cells exposed to 5% organic solvents was used as template for rep-PCR, using REP 1R-Dt and REP 2-Dt, BOXA 1R and $(GTG)_5$ primers. The annealing temperature was 40°C for REP 1R-Dt and REP 2-Dt primers, and 50°C for BOXA 1R and $(GTG)_5$ primers. REP 1R-Dt and REP 2-Dt primers produced the most complex amplified banding pattern for *S. marcescens* IBB_{Po15} cells, with sizes ranging from 300 to 4000 bp (Fig. 4C). Fragments with sizes ranging from 250 to 2000 bp were detected when BOXA 1R and $(GTG)_5$ primers were used. Significant modifications of the rep-PCR patterns were not observed in DNA extracted from *S. marcescens* IBB_{Po15} cells exposed to cyclohexane, *n*-hexane, *n*-decane, toluene, styrene, and ethylbenzene when primers for rep-PCR were used.

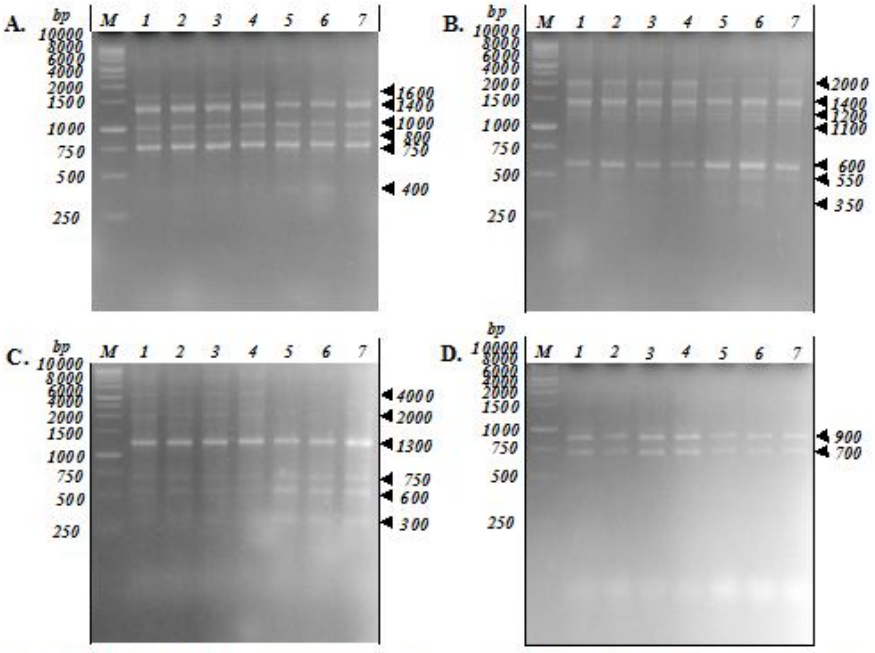

Figure 4 RAPD, rep-PCR and ARDRA patterns of *S. marcescens* IBB_{Po15} cells after 5% organic solvents exposure. Control (*1*), cyclohexane (*2*), *n*-hexane (*3*), *n*-decane (*4*), toluene (*5*), styrene (*6*), ethylbenzene (*7*); 1 kb DNA ladder, Promega (*M*). (**A**) RAPD, AP5 primer, annealing temperature of 36°C, fragments size 400-1600 bp. (**B**) RAPD, AP12 primer, annealing temperature of 36°C, fragments size 350-2000 bp. (**C**) rep-PCR, REP 1R-Dt and REP 2-Dt primers, annealing temperature of 40°C, fragments size 300-4000 bp. (**D**) ARDRA, 27f and 1492r primers, annealing temperature of 55°C, 16S rRNA gene fragment was digested by *Eco*RI at 37°C, fragments size 700 and 900 bp. The detected fragments size has been marked by arrows.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The genomic DNA extracted from *S. marcescens* IBB_{Po15} control cells and from cells exposed to 5% organic solvents was used as template for PCR amplification of 16S rRNA gene, using primers 27f and 1492r. The annealing temperature was 55°C for these primers. The PCR product, fragment with size 1465 bp was then digested at 37°C for 3 h by *Alu*I, *Eco*RI, *Hae*III and *Hha*I restriction endonuclease. When PCR product was digested by *Alu*I or *Eco*RI, two distinct bands (i.e., 200+400 bp, 700+900 bp) were obtained for *S. marcescens* IBB_{Po15} control cells; one distinct band (150 bp) was achieved when the PCR product was digested by both *Hae*III and *Hha*I restriction endonuclease. No significant differences were observed between ARDRA patterns of *S. marcescens* IBB_{Po15} control cell and cells exposed to cyclohexane, *n*-hexane, *n*-decane, toluene, styrene, and ethylbenzene (Fig. 4D).

Polymerase Chain Reaction (PCR)

Genomic DNA extracted from *S. marcescens* IBB_{Po15} control cells and from cells exposed to 5%

organic solvents was used as template for PCR amplification of *ompF*, *HAE1*, *acrAB* transporter genes and *recA* gene. The annealing temperature was 50°C when primers for *ompF*, *HAE1* and *acrAB* genes were used for PCR amplification, and 55°C for *recA* gene primers. The expected PCR products sizes were 209 bp for *ompF*, 550 bp for *HAE1*, 471-494/537-560 bp for *acrAB* genes, and 323 bp for *recA* gene. As could be observed in Fig. 5A-5D, only *HAE1* and *acrAB* genes were detected in the genome of *S. marcescens* IBB_{Po15} control cells. Nevertheless the used primers (i.e., ompF-f, ompF-r; A24f2, A577r2; acrA-f, acrB-r; recA-f, recA-r) are specific for *ompF* [6], *HAE1* [23], *acrAB* [7] and *recA* [24] genes formerly described, they were not specially designed for the bacteria investigated in this study causing unspecific amplification of other fragments.

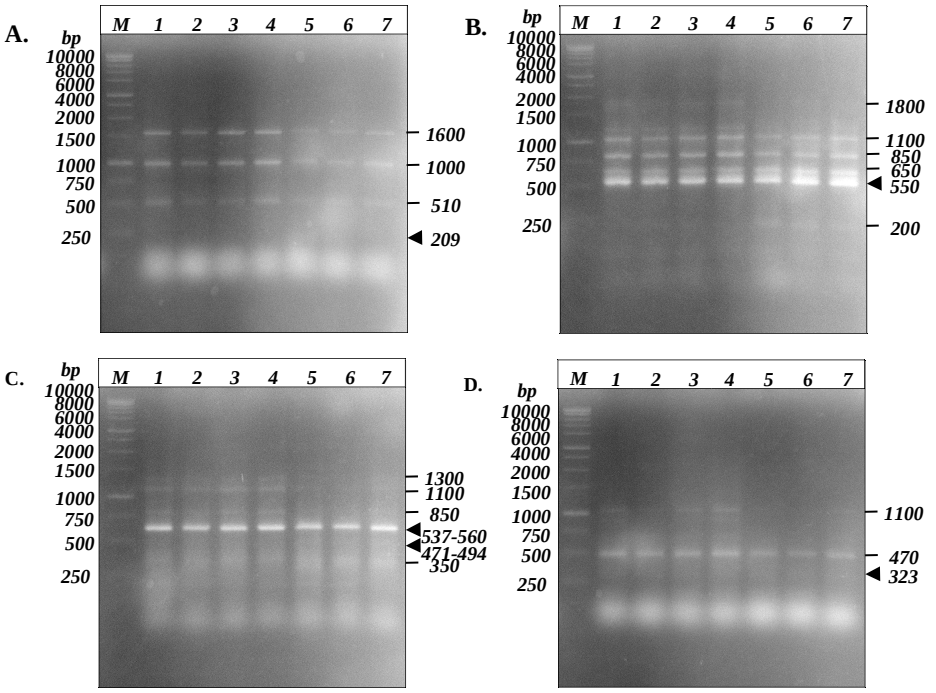

Figure 5 Detection of *ompF*, *HAE1*, *acrAB*, and *recA* genes in DNA extracted from *S. marcescens* IBB_{Po15} cells after 5% organic solvents exposure. Control (***1***), cyclohexane (***2***), *n*-hexane (***3***), *n*-decane (***4***), toluene (***5***), styrene (***6***), ethylbenzene (***7***); 1 kb DNA ladder, Promega (***M***). (**A**) PCR of *ompF* gene, ompF-f and ompF-r primers, annealing temperature of 50°C, expected fragment size 209 bp. (**B**) PCR of *HAE1* gene, A24f2 and A577r2 primers, annealing temperature of 50°C, expected fragment size 550 bp. (**C**) PCR of *acrAB* gene, acrA-f and acrB-r primers, annealing temperature of 50°C, expected fragment size 471-494/537-560. (**D**) PCR of *recA* gene, recA-f and recA-r primers, annealing temperature of 55°C, expected fragment size 323 bp. The expected fragments size has been marked by arrows.

Unspecific amplification of other fragments with sizes ranging from 200 to 1800 bp was acquired when the primers for *ompF*, *HAE1*, *acrAB* and *recA* genes were used (Fig. 5A-5D). No significant modifications of the PCR patterns were observed in DNA extracted from *S. marcescens* IBB_{Po15} cells exposed to cyclohexane, *n*-hexane, *n*-decane, toluene, styrene, and ethylbenzene, as compared with the control, when primers for *HAE1*, and *acrAB* genes were used. When the primers for *ompF* genes were used, three distinct bands (i.e., 510+1000+1600 bp) were acquired for *S. marcescens* IBB_{Po15} control and cyclohexane, *n*-hexane or *n*-decane exposed cells, while in toluene, styrene, and ethylbenzene exposed cells these bands were detected only in barely quantities. When the primers for *recA* genes were used, two distinct bands (i.e., 470+1100 bp) were acquired for *S. marcescens* IBB_{Po15} control and *n*-hexane or *n*-decane exposed cells, while in cyclohexane, toluene, styrene, and ethylbenzene exposed cells the 1100 bp band was detected only in barely quantities. Because the *recA* gene is ubiquitous and highly conserved among all

bacteria examined, this gene was proposed as a phylogenetic marker for distantly related species [33]. The expression of *recA* gene in bacteria is inducible by agents which cause DNA damage [11]. Therefore, significant modifications of the RAPD, ARDRA, rep-PCR and PCR patterns were not observed in DNA extracted from *S. marcescens* IBB_{Po15} cells exposed to 5% organic solvents. On the contrary, modifications of the RAPD, ARDRA, rep-PCR and PCR patterns were observed when *Shewanella putrefaciens* IBB_{Po6}, another Gram-negative bacterium, was exposed to 5% organic solvents [28]. In DNA extracted from *Shewanella putrefaciens* IBB_{Po6} only *HAE1* gene was detected, while *ompF*, *acrAB* and recA genes were not detected and no unspecific amplification were observed. These results were not surprising, because is well know that the *S. marcescens* DNA repair gene is unique in comparison to other Gram-negative bacteria [34].

CONCLUSIONS

S. marcescens IBB_{Po15} (KT315653) which was isolated from an oily sludge sample, exhibited remarkable solvent-tolerance, being able to survive in the presence of high concentrations (40-100%) of toxic organic solvents, such as cyclohexane, *n*-hexane, *n*-decane, toluene, styrene, and ethylbenzene, indicates its ability to survive in solvent rich contaminated sites. Cyclohexane (log P_{OW} = 3.35), *n*-hexane (log P_{OW} = 3.86) and *n*-decane (log P_{OW} = 5.98) were less toxic for *S. marcescens* IBB_{Po15} cells, as compared with toluene (with log P_{OW} = 2.64), styrene (log P_{OW} = 2.86) and ethylbenzene (log P_{OW} = 3.17). Like other *S. marcescens* strains, *S. marcescens* IBB_{Po15} produced extracellular protease and the enzyme production decreased in cells exposed to 5% cyclohexane, *n*-hexane, toluene, styrene, and ethylbenzene. Protease production was unaffected when the cells were exposed to 5% *n*-decane. The release of nucleic acids was higher when *S. marcescens* IBB_{Po15} cells were exposed to 5% cyclohexane, *n*-hexane, ethylbenzene, toluene, styrene, as compared with their release from control and *n*-decane exposed cells. Results presented here indicated that *S. marcescens* IBB_{Po15} cells produced carotenoid pigments, and alteration of the phytoene and lycopene were observed in cyclohexane, *n*-hexane, *n*-decane, toluene, styrene, and ethylbenzene exposed cells. Furthermore, the exposure of *S. marcescens* IBB_{Po15} cells to these toxic organic solvents induced also changes in the extracellular (including the 50 kDa protein) and intracellular (including the 39, 41, 43, 53, 110 kDa proteins) proteins profile. However, only *HAE1* and *acrAB* genes were detected in the genome of *S. marcescens* IBB_{Po15} cells, the unspecific amplification of other fragments with sizes ranging from 470 to1600 bp being acquired when the primers for *ompF* and *recA* genes were used. Considerable RAPD, ARDRA, rep-PCR and PCR pattern modifications were not observed in DNA extracted from *S. marcescens* IBB_{Po15} cells exposed to 5% cyclohexane, *n*-hexane, *n*-decane, toluene, styrene, and ethylbenzene, as compared with the control cells maybe due to their DNA repair gene which are well known to be unique in comparison with other bacteria.

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