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Preliminary study of the properties of the plasmid pSym of the species *Rhizobium sullae* by the application of a bacterial conjugation with the species of *Pseudomonas aeruginosa*

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ABSTRACT

Background: The species *R. sullae*, the microsymbiant specific to the *H. coronarium* L. plant, is able to produce exopolysaccharides (EPS) which play an important role in nodulation and protection of the bacteria against environmental stress. **Objective:** Most of the genes contributing to the nodulation and encoding the EPS are located on the symbiotic plasmid pSym of *Rhizobium*. The main objectives of this work are to study the properties of these genes and to determine the role of EPS products. The present work consists of carrying out a transfer by conjugation of the symbiotic plasmid of the *R. sullae* strain towards the *Pseudomonas aeruginosa* strain on solid culture medium and to study the characters acquired by the transconjugants. **Results** The use of the chloramphenicol antibiotic and the calcofluor white as selection agents have made it possible to select transconjugant strains capable to produce the exopolysaccharides. The determination of the minimum inhibitory concentration to heavy metals has revealed a considerable resistance of the transconjugant strains to mercury chloride, which can withstand concentrations up to 1250 µg / ml, in contrast to the metal-sensitive recipient strain (MIC = 200 µg / ml). The nodulation capacity was tested with the three types of strains; the donor bacteria (*R. sullae*) showed their infectivity by the formation of the nodules, whereas the recipient strain (*Pseudomonas aeruginosa*) and the transconjugant strains were non-infectives. **Conclusion:** Thus it is important to establish a relation between the production of EPS and the resistance of the bacteria to certain environmental stresses, in particular resistance to heavy metals. The horizontal transfer of genes is one of the strategies that contribute to the generation of genetic variations in the environment have changed the perception of aspects of biology.

KEYWORDS: *R. sullae*, symbiotic plasmid, Conjugative transfer, resistance to heavy metals, symbiosis.

INTRODUCTION

Legume plants are able to enter into symbiosis with bacteria belonging to the family *Rhizobiaceae* [45]. The species *Rhizobium sullae* is the specific partner of the forage legume *H. coronrium* L. (sulla) [39]. The ability of legumes to engage in a dinitrogen fixing symbiosis with soil bacteria, make their culture important in productivity agriculture [21].

The symbiosis *Rhizobium*-legume is specific and is governed by signaling molecules produced from both host and bacteria [36]. The establishing of a symbiotic nitrogen fixation is important for the plant especially when the plant is under conditions of nutrient deficiency. This process occurs in the roots of the leguminous plants within specialized organs called nodules [29, 23]. Understanding and then mastering the processes leading to the establishment of symbiosis are a major step towards the development of a less polluting agriculture [3].

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In addition to flavonoids and Nod factors, surface polysaccharides (lipopolysaccharides (LPS), capsular polysaccharides (KPS), exopolysaccharides (EPS) can play a significant role in the nodulation process [10]. EPS produced by rhizobia are essential for the establishment of symbiotic nitrogen fixation with legumes and are considered as signaling molecules [14, 10]. EPS play an essential role in the formation of the infection thread and in root nodule development [33]. Bacterial EPS has been shown to provide protection from such environmental insults as desiccation, predation, and the effects of antibiotics [6, 40].

The production of EPS by the species *R. sullae* in the presence of different carbon sources is highlighted by Gharzouli et al., in 2012 [11]. *R.sullae* is characterized by the production of high molecular weight and low molecular weight EPS, the latter having a structure rich in fucose (30%), which increases the nodulation efficiency in the species. Thus, the production of large amounts of high molecular weight EPS increases the resistance of the bacteria to drought conditions [12].

The genomes of fast-growing rhizobia usually consist of circular chromosome and one or more large plasmids containing genes required for establishing symbiosis. These plasmids are commonly known as the pSyms. They contain functionally important genes contributing to competitive fitness of this group of bacteria in the rhizosphere, including nodulation competitiveness, exopolysaccharides and lipopolysaccharides synthesis, utilization of different carbon source and plasmids transfer. Conjugative transfer of these large plasmids thus plays an important role in the evolution of bacteria species [7].The conjugation of the rhizobial plasmids was carried out with the symbiotic plasmids as a means to study their functions and their evolutions [43].

Pseudomonase aeruginosa is the most widely known and distributed species of the genus *Pseudomonase*, this bacteria is wide spread in nature, is ubiquitous and normally lives in the saprophytic state, exists in water, soils and plants [46]. It's morphological and biochemical characteristics are well studied. It has a large genome, relatively well preserved and totally sequenced [37]. *P. aeruginosa* is a microorganism naturally resistant to a large number of antibiotics, which has long been attributed to an impermeability of its outer membrane to the antimicrobial molecules [22]. However, the discovery of the first constitutive efflux pump in this bacterium (MexAB-OPrM) has shown that *P. aeruginosa's* natural resistance results largely from the expression of efflux systems [27]. For these reasons this species is used as a recipient bacterium in our study.

The aim of our study is to demonstrate the role of genes carried by the symbiotic plasmid of strains of the species *R. sullae* and to determine other properties of the exopolysaccharides produced by this species by the application of a bacterial conjugation with *P. aeruginosa*. Selection of transconjugants by chloramphenicol and calcofluor white allowed us to reveal transconjugated *P. aeruginosa* cells capable of producing rhizobial EPS. The transconjugants showed significant resistance to mercury chloride. The selected bacteria are unable to infect the roots of the legume plant *H. coronrium* L. (sulla).

MATERIALS AND METHODS

Media and bacterial growth:

The bacterial strains used in this study belong to tow varieties of *R. sullae*: *R. sullae* A6 (collected in Constantine, Algeria), and *R. sullae* RHF (collected Pisa, Italy) and another strain of the species *Pseudomonas aeruginosa* (Constantine, Algeria). Strains of *R. sullae* were grown on the yeast extract mannitol agar (YMA) solid medium (mannitol, 10 g/liter; KH_2PO_4, 0.5 g/liter; $MgSO_4 \cdot 7H_2O$, 0.2 g/liter; NaCl, 0.1 g/liter; yeast extract, 0.5 g/liter; agar, 15 g/liter), on the Tryptone-yeast (TY) broth medium (Tryptone, 5 g/liter; yeast extract, 3 g/liter; $CaCl_2.H_2O$, 0.87g/liter), or Tryptone- yeast agar (TYA) solid medium (TY supplemented with 12g of agar in liter) at 28°C for 24h. Strain of *Pseudomonas aeruginosa* is grown on the medium of King A (gelatine peptone (pancreatic), 20 g/liter; magnesium chloride, 1.4 g/liter; potassium sulfate, 10 g/liter; agar, 15 g/liter), on the medium of King B (mixed peptone, 20 g/liter; dipotassium hydrogen phosphate, 1.5 g/liter; magnesium sulfate, 1.5 g/liter; agar, 10 g/liter) and on the nutrient broth (peptone, 15 g/liter; sodium chloride, 6 g/liter; yeast extract, 3 g/liter; D(+)-glucose, 1 g/liter) at 37°C for 24h.

Bacterial conjugation:

Strains of *R. sullae* were grown on TY broth medium at 28°C overnight with shaking. 1ml of this culture is used to inoculate 50ml of TY broth medium. After 4h of incubation in the same conditions, 100µl of culture are taken from an Eppendorf tube for conjugation. Strain of *Pseudomonas aeruginosa* is grown on nutrient broth at 37°C for 24h with shaking, 100µl of culture are taken from an Eppendorf tube for conjugation. The tow tubes are centrifuged at 1200 rpm for 1min. Resuspended each pellet in 100µl of TY broth medium, then harvested in the same tube. The mixture is deposited on a sterile nitrocellulose membrane which rests on the surface of a Petri dish containing the TYA medium. A dish is incubated at 30°C overnight.

Subsequently the contents of the membrane are transferred into an Eppendorf tube containing 1 ml of the sterile physiological water. A dilution series of 10^{-1} to 10^{-5} is carried out. A volume of 100 µl of each dilution is spread on Petri dishes containing the selective medium for the transconjugants. The TYA medium containing

chloramphenicol at a concentration of 4000µg / ml and 5000µg / ml. The colonies obtained are spread on the YMA medium containing 0.02% of Calcofluor white. Colonies that resist chloramphenicol and appear brilliant under UV in the presence of calcofluor are selected. The selected transconjugants are noted: PA1, PA2, TF1, TF2.

Resistance to heavy metals:

The minimum inhibitory concentration (MICs) is determined by cultivating the different strains (donor, recipient and transconjugant) on TYA medium containing different concentrations of: Mercury chloride ($HgCl_2$), Zinc chloride ($ZnCl_2$), and antimony (SbO_3) from 100 to 9000 µg / ml. The incubation lasts 3 to 7 days at 30 ° C.

Plant assays:

Seeds of *H. coronarium* L. were surface sterilized and germinated as described by Vincent [44]. Germinated seeds were aseptically planted in modified Leonard's bottle-jar containing the sand-vermiculite mixture watered with a Fahraeus nutrition solution [8]. Ten plants were then inoculated with 2 ml of exponentially grown bacteria. Pots were placed at room temperature where it was possible to modulate the light conditions for 6 to 8weeks.

In order to test the infectivity of all strains (donor, recipient and transconjugant), the seeds are inoculated with the selected transcanjugate bacteria, inoculated with *Pseudomonas aerugenosa* strain as a negative control, and a third group with the strains of *R.sullae* as positive control.

RESULTS AND DISCUSSION

Phenotype of colonies:

The strains of *R. sullae* (A6 and RHF) on sold medium (YMA) and after 48h of incubation give colonies of circular, convex shape, slightly raised with creamy texture. They are translucent, transparent or opaque and mucilaginous. This aspect corresponds to the characteristics of the bacteria of *Rhizobium* genus [19]. The colonies of the *Pseudomonas aeruginosa* strain on king A and B after incubation for 48 h at 37 ° C appear small, dry, rough, round, convex and smooth [31].

The selection of transconjugants:

Sold medium TYA with antibiotic is one of the most widely used media for the selection of transconjugants [43]. Resistance to antibiotics is frequently used in rhizobial studies as a means of identifying strains and is also considered a good factor in comparing different strains [2].

The culture on the sold medium TYA supplemented with chloramphenicol at a concentration of 4000 µg / ml allows the growth of strains of *R. sullae* (A6 and RHF), only the *Pseudomonas* strain and the transconjugants which are able to grow at a concentration of 5000 µg / ml.

Chloramphenicol is a bacteriostatic agent that inhibits protein synthesis. *P. aeruginosa* is usually intrinsically resistant to chloramphenicol, in part due to the MexAB-OprM efflux system [28].

The colonies of which appear on the TYA medium supplemented with 5000 µg / ml of chloramphenicol are tested on the sold medium YMA supplemented with 0.02% of calcofluor white. Calcofluor white is a fluorescent dye under ultraviolet (UV) light, which allows the selection of strains producing exopolysaccharide (EPS) [18].

Some colonies showed a fluorescent and viscous appearance comparable to the colonies of *Rhisobium sullae* (A6 and RHF). On the other hand, strain of *Pseudomonas aeruginosa*, which is used as a control strain, gives dry and rough colonies in the presence of calcofluor white (Fig.1). This suggests that a conjugative transfer of plasmid DNA to the *Pseudomonas aeruginosa* strains is performed. The viscous colonies are selected to test their resistance to heavy metals and their ability to nodulate *Hedysarum coronarium* L. plant (Sulla), the macrosymbiant specific to the bacteria of *R. sullae*. Four transconjugants strains were selected: TF 1 and TF 2 (derived from conjugation with the RHF strain) and PA 1 and PA 2 (derived from conjugation with strain A6).

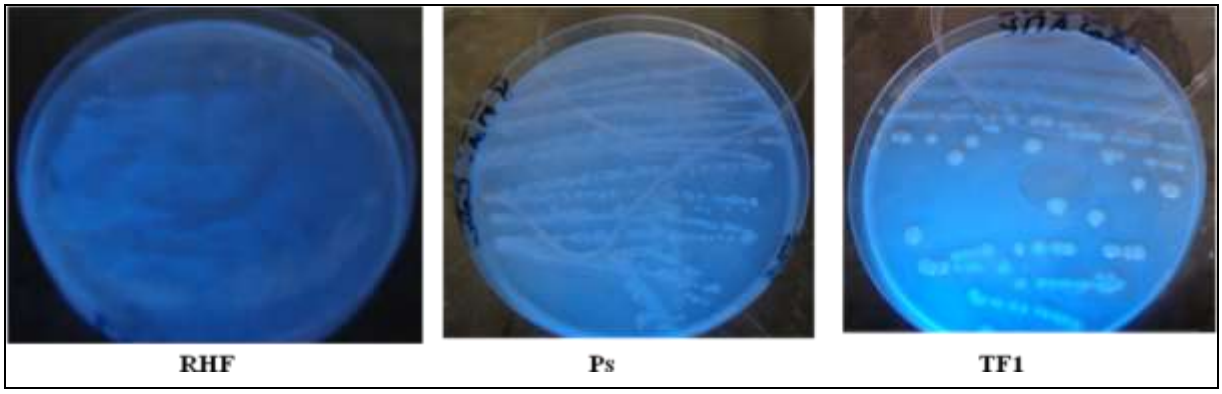

Fig. 1: Appearance of the colonies under UV on YMA medium containing Calcofluor white. RHF : *R. sullae* RHF, Ps : *Pseudomonas aeruginosa*, TF1 : transconjugant starin.

In order to verify the purification of the obtained strains, coloring Gram is carried out. Microscopic observation revealed that the *Pseudomonas aeruginosa* strain and the transconjugant showed a similar appearance by the appearance of Gram-negative pink bacilli. It is a long and fine bacillus with polar mobility. While the *R. sullae* strains reveals small pink sticks.

The production of EPS is specific to rhizobia, which gives a viscosity to bacterial colonies when they are cultivated on a solid medium [30]. Strains defective in production of EPS give small, opaque, and non-mucoid colonies on YMA [20, 18].

The genes coding for the biosynthesis of EPS (*exo* / *exs* or *pss*) form large groups distributed between the genome and the symbiotic plasmid (pSymB) [38]. These genes encode the synthesis of the two types of EPS: succinoglycans (EPSI) and galactoglycans (EPSII). The biosynthesis of EPS I is directed by the product of the *exo* genes, whereas EPS II is produced by the enzymes encoded by the *exp* gene [20].

EPS constitute a layer around the cell surface and protects bacteria against abiotic stress [34]. These molecules play a role against desiccation and confer resistance to bacteriophages [10]. The *R. sullae* (A6 and RHF) strains secrete low and high molecular weight EPS which play an important role in resistance to drought conditions [12].

Resistance to heavy metals:

The minimum inhibitory concentrations (MICs) are determined after incubation on TYA in presence of heavy metals at different concentrations (Fig. 2). The results obtained show that all the transconjugants strains identified tolerates mercury better than the donor strains (*R. sullae* A6 and RHF) and the recipient strains (*Pseudomonas aeruginosa*) (Table 1).

Table 1: The minimal inhibitory concentrations (MIC) (µg / ml) of strains (donor, receptor, transconjugant) grown on TY in presence of different heavy metals.

	A6	RHF	Ps	PA1	PA2	TF1	TF2
$HgCl_2$	500	800	200	1250	1250	1000	1000
$ZnCl_2$	3500	3500	3500	3500	3500	3500	3500
SbO_3	7000	7000	7000	7000	7000	7000	7000

Struffi Struffi et al. [41] showed the sensitivity of *Rhizobium sullae* to mercury. The work of Maris [25] showed that the *P. aeruginosa* strain resists cetrimide while it is sensitive to mercury chloride. The EPS can play a role in heavy metal entrapment [33].

Fig. 2: Resistance of donor strains (*R. sullae* RHF « F », R. sullae A6 « A6 »), recepient strains (*P. aeruginosa* « Ps ») and transconjugants strains (PA1 et PA2) at the mercury at different concentration.

Horizontal gene transfer plays an instrumental role in prokaryotic evolution because it facilitates the rapid acquisition of complex phenotypic traits required for pathogenicity, symbiosis, metabolism, fitness, and antimicrobial resistance [17].

The toxic effects of heavy metals on soil micro-organisms depend on their biological availability. Microorganisms have developed several mechanisms to immobilize, mobilize or transform the effects of heavy metals. These resistance mechanisms are encoded by plasmid and transposon genes and possibly by the transfer of genes or spontaneous mutations that allow the bacteria to acquire resistance to heavy metals [13]. The resistance of soil bacteria to heavy metals is very essential for the growth of plants, especially legumes.

Toxic metals are known as potent inhibitors of several enzymatic activities. Microorganisms, however, have a high capacity to adapt to hostile environments by the appearance of resistant strains. Thus toxic mercury Hg^{++} can be reduced to Hg(O), non-toxic volatile form. This function is coded by plasmids of high molecular weight [1].

Mercury chloride is among heavy metals that are exclusively toxic even at low amounts and it has no biological interest [26]. Several tons of mercury is released into the environment by the chemical and mining industries, polluting water and soil in an important way. This metal, which can be integrated into the food chain, is very toxic, especially in the form of methylmercury, for humans and animals. Natural or transgenic bacteria that resist high levels of mercury through the mercuri-reductase enzyme can be used as an alternative environmental cleaner [4].

Sustainability and environmental safety of agricultural production relies on eco-friendly approaches like biofertilizers, biopesticides and crop residue return [16]. The biological depollution by using of microorganisms to modify the speciation of metals is one of the most important methods of managing environments contaminated with heavy metals [26].

Bacterial conjugation is one of the most widely used mechanisms for studying the phenomenon of resistance (antibiotics or heavy metals) in bacteria. It is often manipulated to determine the role of certain chromosomal or plasmid genes.

Plant assays:

After a culture period of 8 weeks in a culture chamber under controlled conditions, the plants are taken to evaluate the formation of the nodules. Plant growth was normal until the end of the test. The results of the nodulation test show that the donor strains are infectious, capable of forming nodules with the Sulla plant, whereas transconjugants are incapable of forming nodules with the host plant (*Hedysarum coronarium* L).

Bacteria of the *Rhizobium* genus have the ability to form symbiosis with plants of the legume family. The nodules are metabolic exchange organs between bacteria and plants [42]. The nitrogen-fixing bacteria nodulating the Sulla plant was isolated and named *Rhizobium sullae* [39].

Exoplolysaccharides produced by rhizobia act as signaling molecules and play an important role in the formation of nodules [38]. The work of Gharzouli et al. [11] showed that when a large quantity of EPS was produced, a good infectivity was observed by the formation of significant number of nodules. Establishing a symbiotic relationship between bacteria and leguminous plants requires a specific communication process that includes the expression of nodulation (*nod*) and nitrogen fixation (*nif*, *fix*, and *fdx*) genes located in the genome of the microsymbiont (chromosome and the symbiotic plasmid) [5]. For this reason the transconjugants obtained remain incapable of nodulating the host plant. Tejerizo et al. [43] reports that sometimes there is a transfer of a non-symbiotic accessory plasmid.

Conclusion:

The production of EPS is a main characteristic of *Rhizobium* genus. The genes involved in the synthesis of EPS form a large group located on the PsymB symbiotic plasmid [9]. The conjugative transfer of symbiotic plasmids has often been used to study their role in the evolution of *Rhizobium*.

In our study we applied a bacterial conjugation transfer on a solid medium containing the agar between the species *R.sullae* and *P. aeruginosa*, from which transconjugant strains capable of producing amounts of EPS detected by calcofluor under Ultraviolet light were onbtained. This gives a smooth, viscous appearance to the bacterial colonies. The acquisition of new versions of genes by the species *P. aeruginosa* has allowed the bacteria to resist large amounts of mercury chloride. This resistance suggests that the production of EPS by transconjugant strains appears to increase their tolerance to heavy metals. Resistance to heavy metals is an important trait for soil bacteria.

Conjugative transfer of plasmids is an important process for both donor and recipient strain. For the first, it allows to determine the properties and functions of the genes carried by its replicon, which will provide new data on the species concerned. Thus the transfer can carry new interesting and evolving characters to the recipient strain. The ability of *Pseudomonas* to produce new molecules such as rhizobial EPS appears to be beneficial to the species. Despite their ability to produce EPS, transconjugant bacteria remain non-infectious for the Sulla legume plant, as they do not have all the genetic material needed for the nodulation process. *Rhizobium*

strains nodulating legumes are considered to be particularly symbiosis-specific because of their genetic characteristics [32, 35].

Nitrogen-fixing symbiosis can be used to increase soil fertility especially for nutrient deficient soils but also to restore ecosystems disturbed or contaminated by toxic compounds such as heavy metals [26].

Use of microbial inoculants or plant growth- promoting rhizobateria (PGPR) for the enhancement of sustainable agricultural production is becoming a more widely accepted practice in intensive agriculture in many parts of the world [24]. Thus species of the genus *Pseudomonas* are among the bacteria with PGPR effect [15], therefore, to select strains of high resistance to heavy metals constitutes an essential agronomic advantage by their application in the biodepollution and fertilization of agricultural soils.

It is very useful to carry out work on symbiosis of legumes-*Rhizobium*, since better understanding of the different key processes can better integrate this symbiosis in crop cycles in order to move towards sustainable development.

Finally, it is necessary to study other properties which can be carried by the symbiotic plasmid of the species *R. sullae* by the application of other approaches such as the bacterial transformation and the insertion of the transposons.

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