A new mutation in the *pufL* gene responsible for the terbutryn resistance phenotype in *Rubrivivax gelatinosus*

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Abstract *Rubrivivax gelatinosus* **is a facultative phototrophic non-sulfur bacterium belonging to the β subclass of the purple bacteria. A terbutryn-resistant mutant of *R. gelatinosus* has been isolated and characterized. Increased resistance levels to terbutryn (300-fold), atrazine (6-fold) and *o*-phenanthroline (3-fold) were observed for the mutant compared with wild type. Sequence analysis of the mutant revealed a new mutation in the *pufL* gene coding for the L subunit of the reaction centre (RC) at codon 192 leading to an amino-acid substitution from Gly in the wild type to Asp in the mutant. This substitution is located in the D helix of the L subunit, suggesting an interaction between terbutryn and this part of the polypeptide in the RC of *R. gelatinosus*. This is the first report of a mutation leading to herbicide resistance and affecting the D helix in purple bacteria. Furthermore *R. gelatinosus* wild type is highly sensitive to *o*-phenanthroline compared with other purple bacteria (*Rhodobacter capsulatus* and *Rhodobacter sphaeroides*). Sequence comparison of the L subunit from six purple bacteria in which *o*-phenanthroline sensitivity was measured suggests that Ser^{L226} might be responsible for this phenotype.**

Key words: Photosynthetic bacterium; Herbicide; Herbicide mutant; Cross-resistance; Reaction center

1. Introduction

The primary photochemical reactions of bacterial photosynthesis take place within a membrane-bound pigment-protein complex known as the photochemical reaction centre (RC). RCs have been isolated from many species of purple bacteria. The best characterized are those from *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* which have been crystallized and the structures of which are known at atomic resolution [1–3]. The RC of purple bacteria is composed of subunits called L, M and H and in some species, includes a tetrahemic cytochrome *c* protruding into the periplasmic space. The pigments are buried in LM core polypeptides, allowing transmembrane, unidirectional electron transfer from the dimer of bacteriochlorophylls (P) to the primary quinone electron acceptor, the quinone (Q_A). Q_A, buried within M polypeptide, is able to accept one electron. The secondary acceptor (Q_B), a quinone from the quinone pool contained in the membrane, is able to accept two electrons. This secondary acceptor is bound in a pocket called the Q_B site localized in the L polypeptide. Q_B affinity to its site depends on its oxidoreduction state; the semiquinone binds tighly to the Q_B site, unlike the oxidized and double reduced forms. Q_B acts as a mobile electron carrier: after accepting two electrons and two protons it dissociates from the

RC and enters the quinone pool. Subsequent steps of proton translocation using the bc1 complex create a pH gradient, which drives ATP synthesis.

Different classes of herbicides have been known for many years to inhibit photosynthesis at the level of the RC by blocking the electron transfer from Q_A^- to Q_B. These herbicides, which also inhibit electron transfer in photosystem II (PSII) in higher plants, green algae and cyanobacteria, act by competing with Q_B for its binding site. The binding site for Q_B is formed by residues located between the D and E α helices of the L subunit of the RC, and in the homologous region of the D1 subunit of the PSII, equivalent to polypeptide L [4]. The inhibitors *o*-phenanthroline and terbutryn were found by X-ray studies to bind within Q_B site in *R. viridis* RC [5,6]. These structural studies confirmed and extended the previous proposals based (1) on photoaffinity labelling with azido-atrazine which binds to the L and D1 subunits [7,8] and (2) on the localization of almost all mutations conferring herbicide resistances which map to the part of genes coding for L and D1 residues located in the Q_B site [9–11].

The genes coding for the subunits of RCs are organized in operons. The nucleotide sequence of the *puf* operon has been determined for five species belonging to the α subclass of purple bacteria and one species *Rubrivivax gelatinosus* belonging to the β subclass [12]. The *puf* operon of *R. gelatinosus*, strain IL144, contains two open reading frames (ORFs) in addition to five photosynthetic genes which have been reported in species belonging to the α subclass. These genes include *pufB*, A, L, M and C coding for the β and α subunits of the B870 light-harvesting protein and for the L, M and cytochrome subunits of the RC complex, respectively.

In this work, a spontaneous mutant of *R. gelatinosus*, strain 1 [13], was selected by its resistance to terbutryn. We describe here its phenotypic characterization and the identification of the mutation at the Q_B site. This mutation, G192D, is the first one which maps in the region of *pufL* corresponding to α-helix D.

2. Materials and methods

2.1. Bacterial strains, plasmid and growth media
R. gelatinosus strain 1 (isolated by Uffen) [13] was grown anaerobically under photoheterotrophic conditions at 32°C in malate medium [14].

Echerichia coli XL1B was used as host for cloning in BlueScript plasmid and was grown at 37°C on LB medium [15] with appropriate antibiotics (ampicilline 100 μg/ml and tetracycline 10 μg/ml).

2.2. Selection of terbutryn-resistant mutant
400 ml of wild-type culture were grown photosynthetically (650 lux) to reach a concentration of 10^9 cells/ml. This culture was supplemented with terbutryn (200 μM) and maintained in the same growth conditions for 4 weeks, then cells were plated on solid medium complemented with

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200 μM terbutryn and incubated in photosynthetic conditions. Single colonies were isolated and regrown in liquid medium in the presence of herbicide. A mutant of R. gelatinosus was isolated by its ability to grow photosynthetically in the presence of 200 μM terbutryn with the same generation time as the wild type without herbicide.

2.3. Chromatophore preparation and spectrophotometric measurements

Chromatophores from R. gelatinosus were prepared by differential centrifugation after disruption of cells with a French press and were then resuspended in 10 mM Tris-HCl (pH 8) buffer. Flash excitation spectroscopy and inhibition of electron transfer were measured as described [16].

2.4. Isolation of genomic DNA

10^{11} cells of R. gelatinosus were collected by centrifugation and resuspended in 8 ml Tris-HCl 10 mM (pH 8), EDTA 1 mM, saccharose 25% and kept at −20°C overnight. Cells were disrupted with lysozyme (0.1 mg/ml, 30 min at 37°C) and SDS (10 mg/ml, 30 min at 70°C). Sodium acetate (1.2 M) was added to the disrupted cells. After incubation for 30 min on ice, genomic DNA was extracted with phenol:chloroform, treated with RNase and precipitated with ethanol.

2.5. Molecular biology techniques

Standard methods were performed, if not otherwise indicated, according to Sambrook et al. [15]. Plasmid DNAs were purified using Qiagen columns (Diagen). PCR products and digested DNA fragments were purified using Genclean kit (Bio. 101).

2.5.1. Cloning of DNA fragments. (1) The BglII-KpnI DNA fragment of 1.4 kbp (fragment A) was cloned using probe A (474 bp) synthesized by PCR amplification according to the nucleic-acid sequence of R. gelatinosus strain IL144 [12] using oligonucleotides 1 and 2. The plasmid (pB1400) containing the fragment A was isolated from a mini library of BglII-KpnI fragments cloned in BlueScript plasmid.

(2) Oligonucleotides 3 and 4 were used to amplify the fragment B (958 bp). This fragment was cloned in BlueScript plasmid (pB958). These two DNA fragments from wild type were used to sequence the pufL gene. (3) DNA fragment C (320 bp) from the mutant was obtained by PCR amplification using oligonucleotides 5 and 6 and cloned in BlueScript plasmid (pB320).

2.5.2. Oligonucleotides

(1) 5′ TCGGGCCGATCTACCTGGG 3′ (pufM 2214–2233)
(2) 5′ CGAGAGCGCGTGGAACGG 3′ (pufM 2668–2687)
(3) 5′ GGCCCCAAGCGCCCATCAG 3′ (pufL 1626–1645)
(4) 5′ CCGGTCGCGGCGGGTGACT 3′ (ORF2 688–707)
(5) 5′ CCCGGCGCACATGCGGCG 3′ (pufL 1724–1742)
(6) 5′ GAGCGGCCACTGACTCCAG 3′ (pufL 2024–2043)

2.5.3. Sequencing of DNA fragments. Sequencing of both strands were performed with pB1400, pB958 and pB320 using the dideoxy chain termination method of Sanger et al. [15] with sequenase (version 2.0 kit, Amersham). Sequences of products of at least three independent PCR reactions were determined.

3. Results

3.1. Selection of a R. gelatinosus terbutryn mutant

Generation times of R. gelatinosus in photosynthetic growth conditions are 4.5 h under 3400 lux and 15 h under 650 lux. R. gelatinosus cells are more sensitive to tertubryn when cells are grown under limitating light, and only culture growing under 650 lux was totally inhibited in the presence of 200 μM of terbutryn. Selection was performed under this light intensity as described in Section 2. The colony selected was called the mutant ter^r and used for further analysis.

3.2. Determination of herbicide-resistance levels

Sensitivity to terbutryn of isolated photosynthetic membranes and cross-resistance to other herbicides were analysed to determine the phenotype of this new mutant ter^r. After one saturating flash, electron transfer was measured by the reduction of the electron donor P$^+$ followed by measurement of the
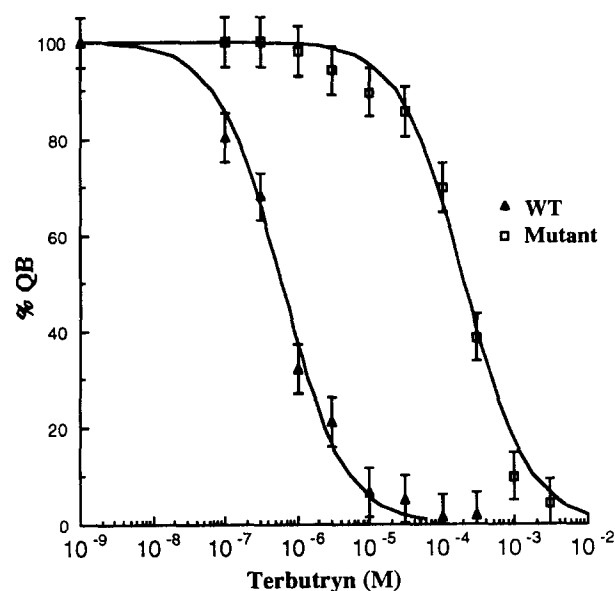

Fig. 1. Inhibition curves for terbutryn in chromatophores from the wild type and the mutant ter^r.

variation of absorption at 785 nm. This reduction is due to the back reactions from $P^+Q_A^-$ to PQ_A and/or from $P^+Q_B^-$ to PQ_B. In the presence of herbicide, P^+ reduction resulted from the $P^+Q_A^-$ recombination in RCs where the Q_B site is occupied by the herbicide and from $P^+Q_B^-$ recombination in RCs where Q_B still occupies its site. Fig. 1 shows the inhibition curves in chromatophores prepared from wild-type bacteria and from the terbutryn-resistant mutant in the presence of various concentrations of terbutryn. Similar experiments were performed using o-phenanthroline and atrazine. The I_{50} values which indicated the concentration needed to block half of the RCs, were determined for the three herbicides. The mutant ($I_{50} = 2 \cdot 10^{-4}$ M) is 300-fold more resistant to terbutryn than the wild type ($I_{50} = 6 \cdot 10^{-7}$ M). For o-phenanthroline, R. gelatinosus wild type is much more sensitive ($I_{50} = 3 \cdot 10^{-6}$ M) than other purple bacteria such as R. capsulatus and R. sphaeroides, $I_{50} = 2 \cdot 10^{-4}$ M [16,17]. The mutant presents a slight cross-resistance, being 3-fold more resistant than the wild type. For atrazine, I_{50} is $4 \cdot 10^{-5}$ M for the wild type. The value of I_{50} for the mutant could not be measured because atrazine precipitated at a concentration higher than 10^{-4} M, before full inhibition was reached. The I_{50} value was estimated from the lower inhibitions to be $\sim 2.5 \cdot 10^{-4}$ M.

3.3. Molecular analysis

3.3.1. Sequence of the pufL gene of R. gelatinosus, strain 1.

The puf operon sequence from R. gelatinosus strain IL144 was published by Nagashima et al. [12]. We used oligonucleotides according to this sequence to clone and sequence the puf operon of strain 1. From this analysis, the organization of the puf operon from R. gelatinosus strain 1 was determined (Fig. 2) and found to be the same as in the IL144 strain. Fragments A and B (Fig. 2) were cloned and sequenced as described in Section 2. Fragment A contains 520 bp of the 3′ region of pufL and the 5′ region of pufM. The upstream region of pufL is contained in fragment B. Fig. 3 represents the pufL nucleotide
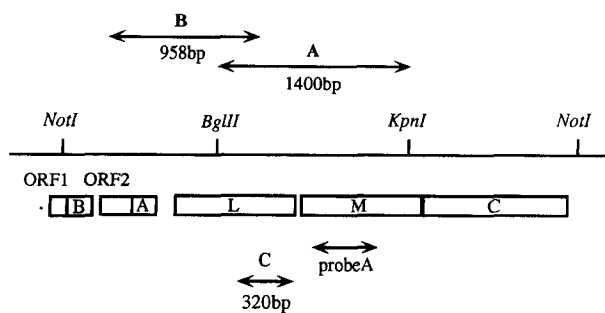
Fig. 2. Gene organization of the *puf* operon from *R. gelatinosus* strain 1. Boxes indicate the position of the genes encoding the α and β subunits of the B870 light harvesting antenna (*puf*A and *puf*B, respectively) and the L, M and cytochrome *c* subunits of the RC (*puf*L, *puf*M and *puf*C, respectively). Two unknown ORFs, ORF1 and ORF2, are indicated. Positions of A–C fragments cloned in (pB1400), (pB958) and (pB320) plasmids, respectively, and probe A are given.

sequence of strain 1. There are 20 nucleotide differences between the two strains. Only one difference gives rise to an amino-acid substitution at position L279 (C-terminus): AAC (Asn) in strain 1 to AAG (Lys) in strain IL144.

3.3.2. Mapping of mutation in puf*L gene of terbutryn-resistant mutant of R. gelatinosus.* As most of the mutations responsible for herbicide resistance phenotypes in purple bacteria were mapped to the quinone-binding site of polypeptide L called 'QB pocket', we investigated this region of *puf*L from the mutant. Fragment C (320 bp) (Figs. 2,3) corresponding to the QB pocket was synthesized by PCR from mutant DNA and sequenced as described in Section 2. Sequencing analysis revealed a point mutation in this region of the *puf*L gene corresponding to a transition (G→A). This mutation results in the substitution of Gly^{L192} by an aspartic acid. Furthermore, this mutation created a new *Bst*YI restriction site. Southern blot analysis of genomic DNA (data not shown) from wild type and
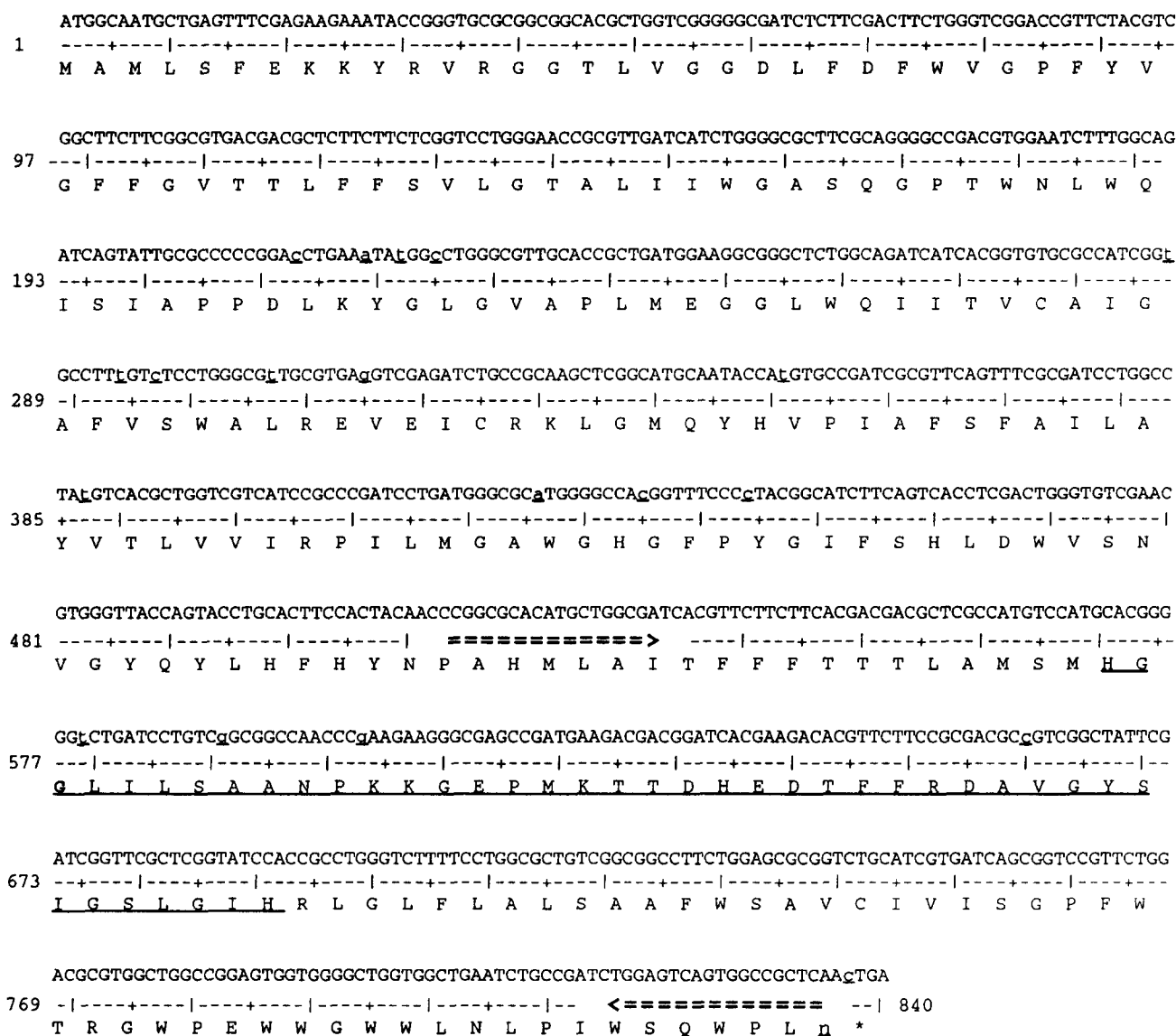

Fig. 3. Nucleotide sequence and the deduced amino-acid sequence of *puf*L gene from *R. gelatinosus* strain 1 (this nucleotide sequence has been submitted to GenBank with accession number U30310). The underlined (lower case) nucleotides and residues are different from those of *R. gelatinosus* strain IL144. The QB pocket (His^{190}-His^{230}) is underlined. Gly^{192} (substituted in the mutant) is in bold. Arrows indicate the oligonucleotides used to amplify a 320-bp fragment from the mutant.

Fig. 4. Amino-acid sequence of the L subunit in the QB-binding region from R. viridis showing in bold all the herbicide mutations described from purple bacteria [6]. Closed box represents DE helix, open boxes represent parts of transmembrane D and E helices. The new mutation affecting the D helix in R. gelatinosus is underlined.

Fig. 6. Amino-acid sequence comparison of the 216–231 region of polypeptide L from six purple bacteria. Thr^{226} is subtitued by Ala and Ser in R. viridis and R. gelatinosus, respectively.

mutant digested by BstYI and hybridized with a radiolabelled probe overlapping the position at 192 confirms in the mutant genomic DNA the presence of the mutation found in the PCR fragment.

4. Discussion

Point mutations within the pufL gene which induce resistance toward herbicides provide information about the binding site of these inhibitors in the L protein.

The new mutation observed in the terbutryn-resistant mutant (ter^r) is located at the codon 192. In ter^r, Gly^{192} was subtituted by Asp, leading to a 300-fold resistance to this herbicide on the acceptor side of RC of R. gelatinosus. This mutation induced slight cross-resistances to other herbicides compared with wild type (6-fold to atrazine and 3-fold to o-phenanthroline).

The Q$_B$ pocket is formed by residues of the L subunit, located in the transmembrane helices D and E, the connecting helix DE and the loop between DE and E. The substitution found in ter^r is located on the D helix of the L subunit. As shown in Fig. 4, all mutations confering herbicide resistances described until now in purple bacteria are located in helix DE, between helices DE and E and in helix E [6,9,18,19]. The mutation we have found in this ter^r mutant is the first one located in this region of the Q$_B$ pocket. This region of L subunit is highly conserved in many purple bacteria and also in the corresponding part of D1 of PSII in organisms performing oxygenic photosynthesis [12,20] (Fig. 5). Its high conservation suggests that it plays an important role in the electron transfer process. It has been poorly studied, probably because no mutation mapping in this region was found in purple bacteria. Only two mutations mapping there were described in cyanobacteria at positions 211 and 219 of D1 [21,22] and one in green algae at position 219 [23].

Why does this L-G192D mutation confer terbutryn resistance? Gly is the smallest residue and is uncharged. It is able

Fig. 5. Amino-acid sequence comparison of a part of D helix of polypeptide L from six purple bacteria and of D1 from oxygenic photosynthetis performing organism [12]. His^{L190} corresponds to His$^{D1\ 215}$. Gly^{L192} from R. gelatinosus is underlined. R. gelatinosus shows three residues 187, 188 and 189 (in relief) different from those of the other purple bacteria (Ro = Roseobacter).

to confer flexibility to a polypeptide chain. Aspartic acid is bulky and charged. Gly^{192} might leave enough space to accommodate binding of terbutryn and aspartic acid might hinder it.

However, it is surprising that this L192 amino acid plays a role in terbutryn-binding if we consider the results obtained with other purple bacteria. The X-ray structure analysis of RC from R. viridis crystallized in the presence of terbutryn has shown that the terbutryn molecule is able to establish at least one hydrogen bond with Ser^{L223} [5]. This result was confirmed by sequencing a terbutryn-resistant mutant of this bacterium bearing the mutation L-S223A [9]. X-ray structure analysis of the RC of this mutant did not show any significant modification of the polypeptide in the Q$_B$ site [9]. In R. sphaeroides, another substitution of Ser^{L223} to Pro also induces resistance to triazine [19]. In addition to this hydrogen bond, it was shown in RC of R. viridis that a second hydrogen bond is possible between the herbicide and the peptide nitrogen of Ile^{224} and that close contacts also exist between Glu^{212}, Ile^{229} and Val^{220} [6].

In the crystallographic RC structure of R. viridis [6], residue L192 is located far from the terbutryn-binding site, too far to be in direct interaction with the herbicide molecule or Q$_B$ atoms. Using the refined coordinates of R. sphaeroides Y RC [3], we found a minimal distance of 10 Å between the Q$_B$ cycle and Gly^{L192} C$_\alpha$. Therefore, the binding site for terbutryn (and perhaps also for Q$_B$) should be different in R. gelatinosus compared with these two other bacteria. One should note that functional properties of Q$_B$ do indeed differ in R. gelatinosus, as shown by pH dependence of P$^+$Q$_B^-$ recombination rate [24]. Besides, comparisons of amino-acid sequences of the helix D region around position 192 from several purple bacteria (Fig. 5) have shown that the most divergent sequence is that of R. gelatinosus, with three important differences at positions 187, 188 and 189. Met, Ser and Met from R. gelatinosus are less hydrophobic and bulkier than Leu, Gly (Ala) and Leu from the other bacteria. In spite of the high conservation between purple bacteria RCs, including R. gelatinosus, which belongs to the β subclass of purple bacteria [12], our results on the ter^r mutant and the different amino-acid sequences of helix D suggest that the structure of this part of the Q$_B$ site of R. gelatinosus RC could be different compared with the RCs already known. The role of helix D will be further investigated using other new mutations.

Characterization of herbicide sensitivities of R. gelatinosus has also shown a high sensitivity of this purple bacterium to o-phenanthroline. R. viridis is the only bacterium which presents a high affinity for this herbicide ($I_{50} = 3 \cdot 10^{-5}$ M) [25], I_{50} values are much higher for R. capsulatus and R. sphaeroides ($I_{50} = 2 \cdot 10^{-4}$ M) [16,17]. An enhanced sensitivity to

o-phenanthroline has also been observed in a *R. capsulatus* mutant bearing the L-T226A mutation ($I_{50} = 5 \cdot 10^{-6}$ M) [16]. Fig. 6 presents a comparison of amino-acid sequences of sensitive and resistant wild-type bacteria. Sensitivity to o-phenanthroline of *R. gelatinosus* supported that the amino acid in position 226 of L subunit is important for the affinity of RC to this herbicide. All strains resistant to o-phenanthroline have a Thr in position 226 and sensitive strains a Ser or an Ala. We intend to replace the S226 of L of *R. gelatinosus* by Thr to test this prediction.

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