A Rifampicin-resistant (*rpoB*) Mutation in *Pseudomonas Stutzeri* Leads to Enhance the Biosynthesis of Secondary Metabolites and Nitrogen Fixation to Adapt Against Harsh Environments

Khandakar Mohiul Alam

Bangladesh Sugarcrop Research Institute

Yongliang Yan

Chinese Academy of Agricultural Sciences

Min Lin

Chinese Academy of Agricultural Sciences

Md. Ariful Islam

Bangladesh Agricultural Research Institute

Ahmed Gaber

Taif University

Akbar Hossain (✉ akbarhossainwrc@gmail.com)

Bangladesh Wheat and Maize Research Institute <https://orcid.org/0000-0003-0264-2712>

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Abstract

The *rpoB* gene encoding for β subunit of RNA polymerase has been targeted as a mutation for importance to Rifampicin Resistant (Rif^r) phenotype of the bacteria. The study aimed to learn the mutational processes, location of the mutation and metabolic capabilities among the strains and nitrogen fixation capacity of the bacteria for acclimatization in the environment. In this study, wild type and 5 *rpoB*Rif^r mutants of *Pseudomonas stutzeri* such Q518R, D521Y, D521V, H531R and I614T were characterized and used as a test system. The robust Rif^r phenotype of *P. Stutzeri* was associated only with base replacements of the amino deposits. Considering these facts, the current study was conducted to investigate the effect of *rpoB* mutants on metabolism, a wild-type and five distinct Rif^r mutants of *P. stutzeri* for utilization of 95 substrates through Biolog GN2 MicroPlates. Several oxidized substrate usage patterns occurred in the Rif^rmutants, use of carboxylic and amino acids significantly increased in various rif^r mutants than that of wild type. The assimilation of carbon and nitrogen sources of rif^r mutants reveals that the organism maintains the adaptation in nutritionally complex environments. Acetylene reduction assay carried out at different times it revealed that considerable variability was found in the nitrogen fixation ability among the studied strains. The highest nitrogenase activity was determined rif^r mutant D521V that was of 2176.6 nmole ethylene/h/mg protein. Base substitution at different sites of rif^rmutatnts caused nucliec acid transition, is responsible for codon change into the amino acids which differ carbon and nitrogen utilization. The assimilation of carbon and nitrogen sources of *Pseudomonas stutzeri* and its rif^r mutants ensures that the organism maintains the adaptation in nutritionally complex environments and can fix nitrogen.

Introduction

The complex of RNA polymerase (RNAP) transcription in the genome is connected with each promoter. Single amino acid substitutions of the organisms can be led to alter gene transcription, physiology and metabolism. Moreover, RNAP is an essential enzyme that is used for controlling pathways in the transcriptional process for gene expression in all living organisms [1]. Replication, transcription and translation processes of RNAP is considered as a major controlling device that can be adjusted the flow of genetic divergence to the numerous cellular tasks needed for detecting and responding to ecological signals. In living organisms like bacteria, RNAP is responsible for synthesizing mRNA, rRNA and tRNA. The 400 kDa RNAP core enzyme consisted of five subunits, included in α -dimer (α_2), β subunit, β' subunit and ω subunit. The majority of catalytic function of RNA polymerase has been conducted by using the β subunit, which is encoded by the *rpoB* gene [2]. The sequencing of *rpoB* gene is a tool that has been used for the molecular characterization of bacteria [3]. Among the core bacterial genes, *rpoB* is a promising marker that has been used for phylogenetic analyses and to know the diversity and identification of bacteria [4].

Rifampicin (Rif) is used as a strong and broad-spectrum antibiotic against pathogenic bacteria. The antibacterial effect of Rif is involved binding the exit channel of RNA polymerase β subunit and

obstructing the elongation of RNA when the transcript becomes 2–3 nt in length [5]. The majority of rif resistant (Rif^r) mutants possess substitutions in RNA polymerase β subunit residues that either make direct contact with Rif or are located near the binding pocket [6]. The rifampin resistance is resulting from mutations of *rpoB* gene leading to specific amino acid alteration in β subunit of RNA polymerase [7]. *Pseudomonas* is playing an important role not only in pathogenicity but also in the development of soil microbial communities [8]. *P. stutzeri* is widely distributed in the natural ecosystem and showed metabolic adaptability, which was consistent with a large population size [9]. Some strains receive particular attention because of specific metabolic capacities as nitrogen fixation, denitrification and degradation of compounds. In order to investigate for novel phenotypes, high throughout metabolic profiling of 96-well format through Biolog GN2 MicroPlates and the mobile monitoring traits of the Omnilog automated plate reader was used in the communication [10]. Using this format was able to find an increased number of phenotypes that were exhibited by various *rpoB* mutants. To our knowledge, no information is available on the position of mutation, substrate utilization capabilities and the mechanisms of transcriptional regulation of the bacteria. In addition, there is no study. There is a limited study where *rpoB* mutants were apparent previously in the wild-type (wt) laboratory strain of *P. stutzeri*. In this study, *rpoB*^{Rif^r} mutants of *P. stutzeri* were characterized to identify the position of mutation, substrate utilization capabilities; and as well as for investigating the transcriptional regulation of metabolic pathways of the bacteria.

Materials And Methods

Separation of Rifampicin-Resistant (Rif^r) A1501 Mutants

Pseudomonas stutzeri A1501 wild type strain was grown in liquid LB medium at 30°C and then 100- μ l aliquots were transferred individually into LB + Rif plates, each plate containing 25, 50 and 75 and 100 μ l /ml of Rif. Spontaneous Rif^r colonies on LB + Rif plates were collected, marked and disinfected 3 times, and stored at -80°C for analysis.

Polymerase Chain Reaction (PCR) of *rpoB* Mutations

cDNA was prepared by using a Puregene kit (Flowgen). The regions contained Rif^r clusters I-III (Nt 879783–883853 of *rpoB*) were amplified from a cDNA template as a 4071 bp PCR product using DNA polymerase (Stratagene). To amplify Rif^r clusters I-III, the used primers were: *rpoB*F, 5' CCTCGGAAGCCACCTACA 3', and *rpoB*R, 5' TTCGATTTGACCCTGGTTT 3'. PCR reactions were conducted in a PTC-200 thermal cycler (MJ Research, Waltham, MA), following 2 min pre-incubation at 95°C. The denaturation cycles were followed by 35 cycles for 1 min at 95°C, annealing for 2 min at 55°C, and elongation for 3 min at 72°C. The PCR products were purified with a Qi-Aquick PCR Purification Kit (QIAGEN) after final incubation for 72 °C. The purified products were sequenced at Tsingke Biotechnology, Beijing. The location of mutations in *rpoB* mutants was identified from multiple sequence

alignments of the PCR products which were performed with the use of the program ClustalW available online through the European Bioinformatics Institute (<http://www.ebi.ac.uk/clustalW>).

rpoB Mutants Cloning

The PCR products were ligated into the T-vector (PMD 19T) and the broad host-vector pLAFR3 using *E. coli* as a host. Rif^r transformant strains were checked for LB + Rif and LB + Cm plates, streak and purified. The plasmids were presented into *P. stutzeri* A1501 from *Escherichia coli* JM109 by triparental conjugation using pRK2013 [11, 12] as an associate plasmid.

Tri-Parental Conjugation

Plasmid PRK 2013 and pLAFR3-*rpoB*, and 1.5 ml each of *P. stutzeri* A1501 were taken in the 3 EP tubes. Then the tubes were centrifuged at a speed of 4000 rpm at 4°C for 10 min. Then 1 ml 0.85% NaCl solution added. The supernatant was discarded and repeated. The ratio of PRK 2013: A1501: pLAFR3-*rpoB* = 2:3:3 were maintained, mixed and centrifuged at a speed of 4000 rpm at 4°C for 10 min. The 3 tubes were mixed together. Again, the bacteria were mixed well and taken 100 ml of bacteria in LB plate and put the bacteria at 30°C for 1 day (24 hours). After 24 hours, the whole amounts of bacteria were taken in a micro-centrifuge tube and added 1 ml NaCl. The serial dilutions were made from 10⁻¹ to 10⁻⁵ and spread on the A15 plates with Tc. The plates were placed at 30°C for 2 days. After 2 days, the plates were observed.

Profiling of Biolog Phenotype

The cultures of the strains were grown overnight in LB plate (wt) or LB + Rif (*rpoB* mutants). The cultures were then diluted into 1 ml GN/GP-IF- inoculating fluid (Gellan gum 0.2 g, NaCl 4 g and Pluronic F-68 0.3 g) for suspending the bacteria in the following day. Cells were diluted into 2 ml GN/GP-IF fluid, and the optical density of the suspensions was adjusted to 52% transmittance by a turbidimeter. Cell suspensions were inoculated (150 µl of suspension per well) into 96-well Biolog Gram-Negative (GN2) MicroPlates. Biolog GN2 microplates were inoculated as defined by the manufacturer instructions and incubated at 30°C for 24 h. Three replicate plates were prepared for each strain. Purple colour development for GN2 plates was assessed after 24 hours of incubation by the Biolog Multiwell Reader. All data were stored for using Biolog software.

Nitrogenase Activity

Bacterial suspensions were overnight incubated in N-free minimum lactated medium at OD₆₀₀ of 0.1 at 30°C under argon atmosphere containing 0.5% oxygen and 10% acetylene for the determination of nitrogenase activity, according to the protocol described by Denoues, et al. [13]. The gas samples were collected periodically and analysed using gas chromatography for the determination of ethylene production. The nitrogenase activity was expressed as nmole ethylene/h/mg protein. Protein concentration was determined using a standard protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin used as the standard.

Statistical Analysis

Data were analysed using Microsoft Excel and Analysis of variance (ANOVA) was performed using the MSTATC software [14]. The differences between treatment means were compared using LSD test at $P \leq 0.05$ [14]. The trend analysis was done by simple linear regression.

Results

Identification of Rifr Mutants

Spontaneous Rifr mutants were selected on LB plates containing 100 µl/ml of Rif and were obtained at a frequency of 1×10^{-9} . All Rifr mutants were strongly resistant > 150 µg rif/ml when grown at the temperature at 30°C on LB agar plates. The isolated mutants expressed a high level of resistance to Rif depending on the localization of mutations in the rpoB sequence. Mutations were mapped to the region of rpoB contained Rifr clusters I-III by transformation and recombination of DNA fragments conferred the resistant phenotype. Results recorded that the population of rifr mutants and wt were 1.2×10^7 and 4.4×10^2 , respectively. According to growth performance, *P. stutzeri* A1501B (expressed rifrpoB gene) grown successfully than the wild type in 10-fold dilution LB + rif plates at 150 µg rif/ml (Fig. 1).

Afterwards, mutations were identifying by sequence analysis. In all cases, the mutations conferring the Rifr phenotype in *P. stutzeri* were found in a highly conserved region of the RNA polymerase β subunit that corresponded to Rifr cluster I and between cluster I and II (Fig. 2).

DNA sequencing of rpoB mutants was executed on a total of 13 isolates. The majority of mutations (12/13) were localized to cluster I, in codons Q518 (1/13), D521 (6/13), and H531 (2/13) (Table 1 and Fig. 2).

Table 1
Summary of the mutational changes of *Pseudomonas stutzeri*.

Codon Position	Codon change	Amino acid change	Representative <i>rpoB</i> alleles
518	CAG to CGG	Q to R	1553
518	CAG to CGG	Q to R	1553
518	CAG to CGG	Q to R	1553
518	CAG to CGG	Q to R	1553
521	GAC to TAC	D to Y	1561
521	GAC to GTC	D to V	1562
521	GAC to GTC	D to V	1562
521	GAC to GTC	D to V	1562
521	GAC to GTC	D to V	1562
521	GAC to GTC	D to V	1562
521	GAC to GTC	D to V	1562
531	CAC to CGC	H to R	1592
531	CAC to CGC	H to R	1592
614	ATC to ACC	I to T	1841

The remainder of Rif^r mutation I614T (1/13) were located in between cluster II and cluster III. No mutations occurred in clusters II or III. Codon D521 (GAC) was a definite hotspot for Rif^R mutations and responsible for 46% of the total; codon Q518 (CAG) was responsible for 30% and codon H531 (CAC) was responsible for 15% of the total. Most of the mutations were found in A-to-T transitions. These A-to-T transitions were situated to 1 hotspot within-cluster I that was accountable for the majority of Rif^r mutations: A1562T in the nucleotide of codon D521, which was resulted in the amino acid change D521V (Fig. 3).

The second most abundant category of mutations was A-to-G transitions. This class of mutations was located exclusively in cluster I as A1553G in the nucleotide of codon Q518, which resulted in the amino acid change Q518R in *rpoB* (Fig. 3). So, we have isolated 13 independent Rif^r mutants of *P. stutzeri*, 12 in cluster I, in codons Q518R (4/13), D521Y (1/13), D521V (5/13), and H531R (2/13). The remainder of one Rif^r mutation I614T (1/13) were located in between cluster II and cluster III. We collected a total of 5 distinct *rpoB* mutations which were Q518R, D521Y, D521V, H531R and I614T. Comparison of site substitutions among *P. stutzeri*, *P. aeruginosa*, *P. putida* and *E. coli* were presented in (Fig. 2).

The *rpoB* gene sequences alignment and the reciprocal amino acids of *P. stutzeri*, *P. aeruginosa*, *P. putida* and *E. coli* are shown. There are several colour coding cluster I for the nucleotides in DNA sequences such

as pink nucleotides which are distinctive to *P. stutzeri*, *P. aeruginosa* and *P. putida*; yellow nucleotides which are similar to *P. stutzeri*, *P. aeruginosa* and *P. putida*; grey nucleotides which are unique to *P. stutzeri* and *P. putida*. Colour coding between cluster I and II for the pink, nucleotides which are distinctive to *P. stutzeri*.

Utilization Patterns of GN2 Microplate Substrates by The Wt and Rif^r Mutants of *P. stutzeri*

Rif^r mutants and wt were inoculated into triplicate in Biolog GN2 MicroPlates. It was incubated in an Omnilog automated plate reader for the determination of the substrate utilization patterns of the bacteria. To determine a baseline for phenotype scoring, the data obtained from all 6 strains in negative control well A1 (Water) were analyzed. It was noticed that the 6 strains exhibited more than 500 arbitrary absorbance units (U) and decided that less than 500 U was considered as a negative signal. Assessing these criteria, it determined that wt strain and 5 Rif^r mutants tested were unable to utilize 71 substrates out of the 95 substrates included in GN2 MicroPlates.

The 24 substrates used by the wt strain could be separated into two broad categories. Eight substrates were strongly used (> 1000 U) and these were comprised of several readily metabolized carboxylic acids (Cis-Aconitic Acid, Citric Acid, β -Hydroxybutyric Acid, Itaconic Acid, α -Keto Glutaric Acid and DL-Lactic Acid) and amino acids (L-Asparagine and L-Glutamic Acid) (Table 2). The remaining 16 substrates were classified by wt (500 to 1000 U), these substrates were included several carboxylic acid, amino acid, carbohydrates and miscellaneous; Dextrin, Tween 80, Bromosuccinic Acid (Table 3).

Table 2
Strongly utilized substrates by the wt and rif^r mutants (> 1000U)

Maximum absorbance value (avg ± SD) ^a							
Substrate	Wt	Q518R	D521V	D521Y	H531R	I614T	LSD < 0.05
D2 Cis-Aconitic Acid	1779 ± 96	1458 ± 7	1686 ± 71	1511 ± 71	1459 ± 23	1364 ± 64	12.02
D3 Citric Acid	1690 ± 67	1240 ± 114	1687 ± 27	1526 ± 65	1351 ± 73	1142 ± 71	137.9
D11β-Hydroxybutyric Acid	1199 ± 80	1223 ± 86	1225 ± 115	1276 ± 63	1359 ± 41	1187 ± 18	144.3
E2 Itaconic Acid	1446 ± 70	948 ± 82	1196 ± 24	961 ± 150	1391 ± 137	840 ± 87	163.8
E4 α-KetoGlutaricAcid	1273 ± 258	1115 ± 89	1094 ± 123	1262 ± 220	1125 ± 158	947 ± 109	272.5
E6 D,L-LacticAcid	1514 ± 51	1300 ± 53	1548 ± 49	1467 ± 49	1577 ± 95	1383 ± 74	126.6
F8 L-Asparagine	1225 ± 84	1417 ± 71	1162 ± 136	1200 ± 88	1195 ± 74	1338 ± 113	183.1
F10 L-Glutamic Acid	1142 ± 31	776 ± 52	850 ± 103	945 ± 123	1223 ± 45	711 ± 66	140.7
^a indicated average values, ± standard deviations. The values shown in boldface were significantly higher than values for the wt by ANOVA (P < 0.05). The values shown in italics were significantly lower than values for the wt by ANOVA (P < 0.05).							

Table 3

Weakly utilized substrates by the wt and rif^r mutants (500 to 1000 U).

Maximum absorbance value (avg $\pm$ SD) ^a							
Substrate	Wt	Q518R	D521V	D521Y	H531R	I614T	LSD < 0.05
A3 Dextrin	673 $\pm$ 35	518 $\pm$ 19	511 $\pm$ 13	410 $\pm$ 39	673 $\pm$ 44	473 $\pm$ 51	67.71
A6 Tween 80	518 $\pm$ 10	636 $\pm$ 62	433 $\pm$ 13	344 $\pm$ 9	380 $\pm$ 66	446 $\pm$ 28	72.32
B6 α -D-Glucose	954 $\pm$ 80	670 $\pm$ 54	1064 $\pm$ 51	999 $\pm$ 64	920 $\pm$ 48	633 $\pm$ 105	135.6
B10 Maltose	785 $\pm$ 141	662 $\pm$ 64	515 $\pm$ 36	421 $\pm$ 59	462 $\pm$ 8	562 $\pm$ 126	126.6
C11 Pyruvic Acid Methyl Ester	990 $\pm$ 65	1210 $\pm$ 33	881 $\pm$ 42	1210 $\pm$ 306	1564 $\pm$ 88	1056 $\pm$ 144	287.4
D7 D-Gluconic Acid	914 $\pm$ 94	495 $\pm$ 18	558 $\pm$ 30	533 $\pm$ 20	585 $\pm$ 97	342 $\pm$ 9	115.5
E7 Malonic Acid	788 $\pm$ 89	736 $\pm$ 73	780 $\pm$ 34	858 $\pm$ 7	876 $\pm$ 24	710 $\pm$ 70	66.79
E8 Propionic Acid	533 $\pm$ 35	478 $\pm$ 110	887 $\pm$ 88	839 $\pm$ 134	1079 $\pm$ 279	422 $\pm$ 85	285.4
E12 Succinic Acid	944 $\pm$ 91	1010 $\pm$ 116	863 $\pm$ 52	864 $\pm$ 61	1050 $\pm$ 183	815 $\pm$ 14	121.2
F1 Bromosuccinic Acid	618 $\pm$ 66	836 $\pm$ 57	586 $\pm$ 10	826 $\pm$ 108	831 $\pm$ 7	745 $\pm$ 110	138.9
F5 D-Alanine	530 $\pm$ 108	366 $\pm$ 30	348 $\pm$ 17	264 $\pm$ 20	535 $\pm$ 18	332 $\pm$ 31	79.08
F6 L-Alanine	572 $\pm$ 34	327 $\pm$ 20	517 $\pm$ 43	331 $\pm$ 14	495 $\pm$ 17	283 $\pm$ 34	58.01
F9 L-Aspartic Acid	501 $\pm$ 66	1104 $\pm$ 98	698 $\pm$ 31	388 $\pm$ 15	598 $\pm$ 194	943 $\pm$ 62	183.1
G3 L-Leucine	653 $\pm$ 115	688 $\pm$ 106	554 $\pm$ 82	630 $\pm$ 116	683 $\pm$ 110	579 $\pm$ 142	222.4
G6 L-Proline	541 $\pm$ 26	470 $\pm$ 197	405 $\pm$ 25	445 $\pm$ 45	656 $\pm$ 22	527 $\pm$ 23	115.9

^a indicated average values, $\pm$ standard deviations. The values shown in boldface were significantly higher than values for the wt by ANOVA ($P < 0.05$). The values shown in italics were significantly lower than values for the wt by ANOVA ($P < 0.05$).

Maximum absorbance value (avg $\pm$ SD) ^a							
G9 L-Serine	649 $\pm$ 73	311 $\pm$ 37	497 $\pm$ 32	446 $\pm$ 41	818 $\pm$ 43	275 $\pm$ 49	67.47
^a indicated average vales, $\pm$ standard deviations. The values shown in boldface were significantly higher than values for the wt by ANOVA (P < 0.05). The values shown in italics were significantly lower than values for the wt by ANOVA (P < 0.05).							

Nitrogenase Activity

Nitrogenase activities were measured among the strains after 4h, 5h, 6h, 7h and 8h. There were found considerable variations of nitrogenase activity among the studied strains. The highest and lowest rate of nitrogenase activity was found of 2176.6 and 1061.2 nmole ethylene/h/mg protein, respectively in rifr mutant D521V and I614T after 7h but at 4h it was 1339.1 and 523.4nmole ethylene/h/mg protein, respectively (Fig. 4). The rifr mutant D521V showed the highest and I614T, the lowest ability of nitrogenase activity in most cases during these intervals. The variations were highly significant after 4h and 5h and; moderately significant after 6h but after 7h and 8h, there was no variation among the strains through the strains Q518R, H531R and I614T showing the increasing trend to nitrogenase activity.

Discussion

Rif^rRpoB Mutants of *P. stutzeri*

The molecular characterization of Rif^r phenotypes revealed that the *rpoB* gene encoding for β subunit of RNA polymerase is a target of these mutations in *E. coli* [15]. The same location-specific mutations are showed in other bacteria such as *Pseudomonas aeruginosa* and *Pseudomonas putida* [8]. In all our studied organisms, the *rpoB* mutations of the bacteria affect residues corresponding to those altered in *E. coli* Rif^r mutants. Here, the results of the analysis of the Rif^r mutants of *P. stutzeri* were presented. The Rif^r clusters of the *rpoB* gene were sequenced and analyzed for 13 mutants of *P. stutzeri*. It is noted that the clusters I-III of the *rpoB* sequence within the Rif^r mutations defined 13 distinct base substitutions at 5 separate sites in *P. stutzeri* (Table 1). Out of the 13 Rif^r mutations sequencing, 5 mutations have consisted of the same A to T transition (Underlined) changing codon D521 (GAC) to V521 (GTC) and its nucleotide position was 1562, 6 mutations were found to consist of the same A to G transition changing codon Q518 (CAG) to R518 (CGG) and H531 (CAC) to R531 (CGC) and its nucleotide position were 1553 and 1592, respectively. Moreover, the single example of the following mutations was found that G to T transition changing codon D521 (GAC) to Y521 (TAC) nucleotide position was 1561 and T to C transition changing codon I614 (ATC) to T614 (ACC) nucleotide position was 1841.

The number of base replacements concerning mutation at different sites varied significantly. It indicated that the neighbouring sequence has a strong effect on the occurrence of these mutations. The most

prominent hotspot of mutation was A:T to G:C transition at the position of 1562 in bacteria [8]. In *E. coli*, the corresponding site locates at the position of 1547, at this position the A: T to G:C substitution is the hotspot in this organism as well [15, 20].

Utilization Patterns of GN2 Microplate Substrates for Phenotype Scoring of Wt and Rif^r Mutants of *P. stutzeri*

High Utilization of Substrates (> 1000 U)

The 5 rif^r mutants and 8 highly utilized substrates produced 40 mutant/substrate combinations of the bacteria (Table 2). From these utilization processes, it was found that 2 examples of remarkably increased in respect of substrate utilization process by the mutants. It found that among others, 19 examples of remarkable decrease and 19 examples of no statistical difference between the wt and the rif^r mutant (Table 2). By contrast, significant differences were noted in the substrate utilization process among the examples. It was noted that in ~ 48% of the cases of (19/40) *rpoB* mutations caused reducing the utilization of substrates normally compared to use strongly by the wt, and in ~ 5% of the cases of (2/40) *rpoB* mutations led to enhance utilization.

The substrate utilization patterns by the *rpoB* mutants could be determined from the presented data. Utilization of Itaconic acid and Glutamic acid were significantly dropped in all rif^r mutants except the H531R mutant (Table 2 and Fig. 5a). On the other hand, utilization of Cis-Aconitic acid and Citric acid were not significantly improved in any rif^r mutants but was diminished significantly by the Q518R, D521Y, H531R and I614T mutations (Table 2 and Fig. 5b). Conversely, utilization of β -Hydroxybutyric acid and L-Asparagine were increased in mutants of H531R and Q518R respectively, but other mutants did not express any significant effect (Table 2 and Fig. 5c). Utilization of α -Keto Glutaric acid and D, L-Lactic acid were significantly lower at the mutants of I614T and Q518R but others had no significant effect of reduction (Table 2).

Weakly Utilized Substrates (500 to 1000 U)

The 5 rif^r mutants and 16 substrates used weakly by the wt resulted in 80 mutants/substrate combinations of the bacteria (Table 3). From these utilization processes, it was found that 13 examples of remarkably increased in substrate utilization by the mutants. It found that compared to 33 examples of remarkably decreased and 34 examples of no statistical difference between the wt and the rif^r mutant. Opposite results were found with strongly utilized substrates. It was found that in ~ 28% of the cases of (13/46) *rpoB* mutations increases the utilization of substrates normally which was used weakly by the wt. The others in 71% of the cases (33/46) of the *rpoB* mutations led to a further reduction in utilization. Mutation to rif^r mutants had no significant effects on the utilization of L-Leucine substrate. Utilization of E12 Succinic acid and G6 L-Proline were decreased considerably by one of the rif^r mutants. On the other hand, propionic and aspartic acid were significantly increased in most of the mutants (Fig. 5 and Fig. 6).

Utilization of Carboxylic Acid and Amino Acids

The utilization of several substrates was significantly improved by rifr mutants' particularly carboxylic acid such as propionic acid, and amino acid as aspartic acid (Table 3 and Fig. 5). This up-regulation process of the substrates utilization was consistent with the increased utilization of β -Hydroxybutyric Acid and L-Asparagine (Table 2 and Fig. 5c). Higher utilization of these substrates was linked with the rifr mutants of H531R, Q518R, D521V, D521Y and I614T. The up-regulation used for multiple substrates by Rifr mutants suggested that a common mechanism of transcriptional regulation of the genes might exist. The utilization of amino acids and carboxylic acids of rifr mutants of *P. putzneri* explains the genes involved in its uptake and utilization. How might rifr mutants play into this? Finally, metabolically versatile *Pseudomonas* usefully utilizes a broad range of organic compounds as carbon and nitrogen sources and follows a different mechanism of catabolite regulation that is involved in a Crc protein that binds mRNA which is acted as a translational repressor. In *Pseudomonas* taxonomic studies [16] revealed that 16S rRNA and ITS1 region represented the *rrn* operon, and the *gyrB* and *rpoB* genes which is interacted with nucleic acid metabolism.

In contrast, the regulation of carbon and nitrogen are involved in two signal transduction systems. The first one is NtrBC, it is a global activator of genes involves in nitrogen assimilation [17] and the second one is CbrAB it plays a global regulatory role in carbon and nitrogen catabolism [18]. The biologic phenotypic analysis in *Pseudomonas fluorescens* SBW25, with activation of CbrAB genes, resulted that in growth defects on 20 carbon sources including seven amino acids (Histidine, proline, leucine, isoleucine, valine, arginine and tyrosine) and four carbohydrates (D-xylose, D-mannose, D-ribose and L-arabitol) [19]. Rifr mutations in the β subunit trouble the interactions among RNA polymerases, regulatory proteins and initiation or termination complexes are well studied [7]. However, it was hypothesized that the exchange in substrate utilization by Rifr mutants may result from different interactions between RNA polymerase and small ligands, regulatory proteins, and regulatory RNA secondary structures [19, 21].

Nitrogenase Activity

Nitrogenase activities were tested at 4, 5, 6, 7 and 8 hours of interval. It was found that the nitrogenase activity was highly significant ($P \leq 0.001$) at 4h and 5h of an interval, at 6h interval was moderately significant ($P \leq 0.050$) and at 7h and 8h interval it was insignificant. At 4h the wild type showed three times higher nitrogenase activity than that of I614T (Fig. 4). Consecutively, the highest nitrogenase activity was determined at 7h interval in rifr mutant D521V that was of 2176.58 nmole ethylene/h/mg proteins and at the same time the lowest was of 1061 ethylene/h/mg protein in I614T. The antibiotic rif is binding to RNA polymerase β subunit within the DNA/RNA channel and acts as an inhibitor blocking the elongation of RNA [8, 22], but there were no nitrogenase inhibitory effects among the studied strains in respect of nitrogen fixation.

Conclusion

Five distinct *rpoB* rifr mutants of *P. stutzeri* of Q518R, D521Y, D521V, H531R and I614T were characterized. Base substitution at different sites of rifr mutants caused nucleic acid transition, is responsible for codon change into the amino acids which differ carbon and nitrogen utilization in the Biolog substrates. It is clearly stated that carboxylic and amino acids serve as good carbon and nitrogen sources with respect to nutrition for the bacteria. The assimilation of carbon and nitrogen sources of *Pseudomonas stutzeri* and its rifr mutants ensures that the organism maintains the adaptation in nutritionally complex environments and can fix nitrogen.

Declarations

Author Contributions

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Declarations

We hereby declare that this manuscript contains no material which has been accepted for the award of any degree or diploma in any university, and that, to the best of our knowledge and belief, the manuscript contains no copy of any material previously published or written by another person except where due reference is made in the text. All authors in the manuscript have approved the submission to ‘Antonie van Leeuwenhoek’.

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Data recorded in the current study are available in all Tables and Figures of the manuscript

Conflict of interest

All the authors have no conflict of interest

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Figure 3

Sequence alignment of the region of RNA polymerase β subunits of *P. stutzeri* containing Rif^r cluster.

Figure 4

Variability of nitrogenase activities in the different *rpoB* mutants and wt type.

Figure 5

The utilization patterns of substrate in wt and Rif^r mutant of *P. stutzeri*. Asterisks are denoted significant differences ($P < 0.05$) from the wt. **(a)** Itaconic acid and Glutamic acid. **(b)** Cis Aconitic acid and Citric acid. **(c)** β -Hydroxybuteric acid and L -Asperagine.

Figure 6

The patterns of substrate utilization in wt and Rif^r mutant of *P. stutzeri*. Asterisks are denoted significant differences ($P \leq 0.05$) from the wt.