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ORIGINAL ARTICLE

Molecular, Physiological and Phenotypic Characterization of *Paracoccus denitrificans* ATCC 19367 Mutant Strain P-87 Producing Improved Coenzyme Q_{10}

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Abstract Coenzyme Q_{10} (CoQ_{10}) is a blockbuster nutraceutical molecule which is often used as an oral supplement in the supportive therapy for cardiovascular diseases, cancer and neurodegenerative diseases. It is commercially produced by fermentation process, hence constructing the high yielding CoQ_{10} producing strains is a pre-requisite for cost effective production. *Paracoccus denitrificans* ATCC 19367, a biochemically versatile organism was selected to carry out the studies on CoQ_{10} yield improvement. The wild type strain was subjected to iterative rounds of mutagenesis using gamma rays and NTG, followed by selection on various inhibitors like CoQ_{10} structural analogues and antibiotics. The screening of mutants were carried out using cane molasses based optimized medium with feeding strategies at shake flask level. In the course of study, the mutant P-87 having marked resistance to gentamicin showed 1.25-fold improvements in specific CoQ_{10} content which was highest among all tested mutant strains. P-87 was phenotypically differentiated from the wild type strain on the basis of carbohydrate assimilation and FAME profile. Molecular differentiation technique based on AFLP profile showed intra specific polymorphism between wild type strain and P-87. This study demonstrated the beneficial outcome of induced mutations leading to gentamicin resistance for improvement of CoQ_{10} production in *P. denitrificans* mutant strain P-87. To investigate the cause of gentamicin resistance, *rpIF* gene from P-87 and wild type was sequenced. No mutations were detected on the *rpIF* partial sequence of P-87; hence gentamicin resistance in P-87 could not be conferred with *rpIF* gene. However, detecting the mutations responsible for gentamicin resistance in P-87 and correlating its role in CoQ_{10} overproduction is essential. Although only 1.25-fold improvement in specific CoQ_{10} content was achieved through mutant P-87, this mutant showed very interesting characteristic, differentiating it from its wild type parent strain *P. denitrificans* ATCC 19367, which are presented in this paper.

Keywords Coenzyme Q_{10} · *Paracoccus denitrificans* · Gamma rays · Gentamicin · AFLP · *rpIF* gene · Strain improvement

Introduction

Coenzyme Q (CoQ: 2,3-dimethoxy-5-methyl-6-multiprenyl-1,4-benzoquinone) molecules are located in the domain of the phospholipid bilayer of cellular membranes. It is involved in the transcriptional regulation of genes, some of which play roles in inflammatory responses, cholesterol metabolism and also demonstrated positive effects on patients suffering from certain cardiovascular conditions, such as conjunctive heart failure and acute myocardial infarction [1–4]. The oral formulations containing coenzyme Q_{10} (CoQ_{10}) have proved to be useful in improving serum CoQ_{10} levels and its cosmetic

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formulations exhibited anti-aging property; therefore there is a great demand for this molecule in the market [5]. The growing demand for CoQ_{10} from the pharmaceutical cosmetic sectors has led to intensified efforts to decrease the cost of CoQ_{10} industrial production. Microbial fermentation using yeasts or bacteria, offers several advantages over chemical and semi-chemical synthesis, including specificity towards all-*trans* biologically active isomer of CoQ_{10} and the reduced production of environmentally hazardous waste [6]. It is produced in naturally high quantities by several microorganisms including strains of gram-negative soil bacteria such as *Rhizobium radiobacter*, *Paracoccus denitrificans*, and *Protomonas extorquens*, as well as some photoautotrophs including *Rhodobacter spheroids*, and yeast species such as *Sporidiobolus johnsonii*, which accumulate intracellular CoQ_{10} to levels between 0.8 and 3.3 mg/g of dry cell weight (DCW) [7]. The selection of suitable strains and further improving the productivity is a prerequisite for cost effective production of CoQ_{10} by fermentation. Hence research efforts on the production of CoQ_{10} by microorganisms are focused on the development of potent strains by conventional mutagenesis and/or metabolic engineering, analysis and modification of the key metabolic pathways, and/or optimization of fermentation strategies. So attempts were made to select the natural CoQ_{10} producing bacterial strain that has not been extensively studied so far.

Paracoccus denitrificans, is known to produce CoQ_{10} under submerged cultivation. Despite the biotechnological potential towards the production of lipid compounds like CoQ_{10}, the low CoQ_{10} content of this natural strain limits its industrial exploitation. Therefore, obtaining hyper producing mutants in order to develop an economic alternative for the production of CoQ_{10} is needed. This strain is not been explored extensively for CoQ_{10} studies like few higher producing bacterial strains Like *R. radiobacter*, and *R. sphaeroides* [8], due to its low CoQ_{10} content and there are no reports on its strain improvement. Hence, the strain improvement study consisting of sequential mutagenesis, rational selection and screening process was undertaken for this strain to bring improvement in CoQ_{10} content. The strains that are developed by induced mutations are genotypically different from their respective parent strains, hence investigation of these changes are essential to characterize them as a novel strains [9–11]. Physiological and phenotypic characteristics as well as molecular differentiation methods like rRNA gene sequences, RAPD, RFLP or AFLP are being used to discriminate the strains of same genera or species and mutants [12–14]. The purpose of this study is to bring significant improvement in CoQ_{10} content of a type strain *P. denitrificans* ATCC 19367 by mutagenesis and rational selection and screening, followed by characterization of the improved strain.

Materials and Methods

Strains, Media and Materials

The bacterial strain *P. denitrificans* ATCC 19367 and its induced mutants were maintained at 4–8 °C on Tryptic soy agar (TSA) slants. A natural variant of the type strain was selected on agar plate containing 13 % cane molasses and used for further studies. All dehydrated media and media components were procured from Hi-Media, India. All solvents (AR grade) were procured from Merck.

Gamma (γ) Rays Induced Mutagenesis

The cell suspension was prepared in saline from the 24 h grown culture on TSA slant to get viable count of 10^9–10^{10} cfu/ml. ^{60}Co was used as a source of γ rays having radiation frequency of 1.5 K γ/h. 10 ml of cell suspension was transferred to sterile falcon tubes and exposed to the irradiation source at different time period in order to get irradiation dose of 25 γ, 50 γ, 100 γ, 250 γ and 400 γ. After exposure the cell suspensions were serially diluted in sterile saline till 10^{-9} dilutions and 0.1 ml of suspensions were spread onto the surface of TSA plates which were incubated at 30 °C for about 96 h. The reduction in viable count was determined as compared to untreated suspension.

N-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) Mutagenesis

The culture was grown overnight in 50 ml of Tryptic Soy Broth (TSB) medium in 500 ml flask at 30 °C, 220 rpm to get viable count of around 10^9–10^{10} cfu/ml. The 20 ml of cell suspension was taken in sterile flask and 500 µl of NTG stock solution (16 mg/ml) was added to get final concentration of 400 µg/ml. The flask was kept with intermittent shaking and 5 ml of sample was taken out at different time intervals. The cells were washed, reconstituted in 5 ml of 10 mM phosphate buffer (pH 7.0) and different dilutions were plated on NA plate to determine the viable count. The reduction in viable count was determined as compared to untreated suspension.

Rational Selection of Mutants

The type strain was tested for its sensitivity towards few structural analogues of CoQ_{10} as well as antibiotics. The most sensitive compounds like menadione, idebenone, gentamicin and vancomycin were used for selection of resistant mutants using gradient plate techniques by incorporating appropriate concentration of compounds. The mutated suspensions showing optimum reduction in

viability were plated on to the inhibitor containing gradient plate for selection of resistant mutant colonies. The resistant colonies appearing towards higher concentration gradient on the plate were picked up and transferred to TSA slants.

Screening of Mutants in Shake Flask

The seed medium contained (g/l): sucrose 60, yeast extract 15, peptone 15, NaCl 5, pH 7.2. The 50 ml seed medium dispensed in 500 ml conical flask was inoculated with working stock culture from 2 to 3 weeks old TSA slants and incubated at 28–30 °C on rotary shaker having 220 rpm, for 24 h. The production medium used for screening of mutants in shake flask contained (g/l): cane molasses 80, $(NH_4)_2{\cdot}SO_4$ 13, K_2HPO_4 0.5, KH_2PO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.25, corn steep liquor (CSL) 40, $CaCO_3$ 20, pH 7.2. The 10 % of grown seed was transferred to 50 ml of production medium in 500 ml conical flask. The production flasks were incubated at 28–30 °C on rotary shaker having 220 rpm for 120 h. The production medium was dosed intermittently at 24 h using para-hydroxy benzoic acid (pHBA) (final concentration 25 mg/l) [15]. Additionally, 5 ml of 30 % sucrose was fed at 48 and 72 h of fermentation cycle [16]. During the screening of mutants, the respective parent strains were included along with mutant strains in shake flask fermentation which served as a control.

Dry Cell Weight (DCW) Measurement

Ten ml of broth was centrifuged at 12,000 rpm for 20 min in a pre-weighed centrifuge tube. The cell mass was quantified by drying at 60 °C until a constant mass was obtained.

CoQ_{10} Extraction Method

The 20 ml of harvested broth was centrifuged at 12,000 rpm for 20 min to get biomass pellet, which was extracted with 20 ml of ethanol by heating in shaking water bath at 60 °C for 3 h. The cells were removed by centrifugation and ethanol layer was re-extracted with 20 ml of hexane. The hexane layer was separated, concentrated till dryness and finally reconstituted with 1 ml of hexane [17].

Quantification of CoQ_{10}

The CoQ_{10} extracted from cell biomass was quantified on HPLC (Agilent 1100) using normal phase Kromasil silica column (250 mm × 4.6 mm, 5 µl particle size) and hexane: isopropyl alcohol (95:5) as mobile phase with a flow rate of 1 ml/min. Detection was carried out at 273 nm [17]. The titer was estimated by comparing the area of sample and standard CoQ_{10} (Sigma, C9538) of known concentration (0.1 mg/ml) and expressed as mg of CoQ_{10} per liter of broth (mg/l). The titer value was divided with DCW to get specific CoQ_{10} content (mg/g of DCW).

Differentiation of Mutant P-87 Based on Carbohydrate Assimilation

Carbohydrate assimilation tests were carried out using Disc Method (Discs from Hi-Media, India). The plates containing phenol red agar base (Ref: M053-100G, HiMedia, India) were surface seeded with 50 µl cell suspension (10^8 cfu/ml) and allowed to dry completely. The carbohydrate discs (Hi-Media, India) were placed and pressed gently on the surface of the plate at sufficient distance (2 cm) from each other. Incubation was carried out at 30 ± 1.0 °C for 18–48 h and observed for the presence of growth around the discs.

Fatty Acid Methyl Ester (FAME) Analysis

Fatty acid profile was obtained for wild type and P-87 by FAME analysis. The bacterial culture was streaked on TSA agar plate by quadrant steak plate method. The plates were incubated at 30 °C for 24 h in an incubator. The loopful (4 mm) of biomass from plates was used for extraction and it was first saponified (NaOH in CH_3OH). Cellular fatty acids were methylated (HCl in CH_3OH), extracted (hexane in methyl *tert*-butyl ether) and cleaned (NaOH) as per following MIDI protocol (http://www.microbialid.com/). The chromatographic analysis was performed using Sherlock method. The 2 µl of extract from 0.5 ml of total extract was injected in gas chromatograph with flame ionization detector along with an auto sampler and an integrator, coupled to a computer system. The Sherlock computer software (version 6.0B; MIDI, Inc.) automatically sets the operating parameters of gas chromatograph each time a sample is processed. The fatty acids were identified from their respective retention time (RT). The fatty acid profile of P-87 was compared with that of wild type strain. Based on the closest match with the FAME profile from MIDI database, the dendrogram was created for wild type and P-87.

Amplified Fragment Length Polymorphism (AFLP)

The bacterial genomic DNA of wild type and P-87 was extracted using ChargeSwitch® gDNA Mini Bacteria Kit (Life Technologies, USA) as per the manufacturer's instructions. Total DNA from both strains were quantified using Qubit® DNA Broad-Range Assay kit with the Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, USA).

AFLP was performed using the AFLP core reagent kit and AFLP Analysis system for microorganisms (Invitrogen, Life Technologies, USA). From each sample 250 ng of genomic DNA was digested simultaneously with MseI/EcoRI enzyme mix (10 units each) at 37 °C for 2 h. Following digestion, EcoRI and MseI adapters (Invitrogen, Life Technologies, USA) were ligated to the ends of the restricted fragments at 20 °C for 2 h. Tenfold diluted ligation mix was prepared in TE buffer. The digested and ligated fragments were pre-amplified with primers pair displaying one selective nucleotide, namely Eco-primer + A (E-A) and Mse-primer + G (M-G) using 20 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 2 min according to the manufacturer's instructions. Different combinations of primers were used for selective amplification (Supplementary Table 1). The pre-amplified DNA of the individual samples from each of strain was further diluted (1:10) and used as the template for selective AFLP amplification. From this diluted pre-amplified solution 2.5 µl of solution was used as template for the selective amplification with five different EcoRI and MseI primer pairs using a 'Touchdown' cycle programmed as follows: One cycle of 94 °C for 30 s; 65 °C for 30 s; and 72 °C for 60 s and further 12 cycles of 94 °C for 30 s; 65 °C (−0.7 °C per cycle) for 30 s; and 72 °C for 1 min, until reaching an optimal annealing temperature of 56 °C, followed by 23 more cycles of 94 °C for 30 s; 56 °C for 30 s and 72 °C for 1 min. All reactions were carried out in a GeneAmp PCR System 9600 (Applied Biosystems, USA) and then stored at 2–6 °C. For loading samples in Applied Biosystems 3130 genetic analyzer, mixture of 0.5 µl selective amplification products, 0.25 µl GenScan 500 ROX, 9.25 µl Hi-di Formamide was prepared for each amplification reaction. The mixture was denatured at 95 °C for 3 min and immediately chilled onto ice. The samples were loaded in machine with required instrument protocol and the results were analyzed using GeneMapper software. For estimation of similarity–coefficient (SC), the formula was used to generate computer algorithm and transformed into genetic distance (GD) analysis profile [18]. The SC was converted into GD using the equation GD = 1–SC. GeneMapper data was exported into Microsoft Excel format and further analyzed to estimate the total number of alleles and polymorphic band using statistical tools.

16S rRNA Sequencing

The bacterial genomic DNA of wild type and P-87 was extracted using ChargeSwitch® gDNA Mini Bacteria Kit (Life Technologies, USA) as per the manufacturer's instructions. Universal bacterial 16S rDNA primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′) were used for amplification. The PCR reaction mixture contained 100–200 ng of genomic DNA, 1X PCR buffer, 2.5 mM of $MgCl_2$, 0.1 mM of each dNTP, 0.2 mM of primer solution, 1.0 unit per reaction of Taq DNA polymerase and Nuclease free water to make total reaction volume of 25 µl. The PCR was performed at 95 °C for 10 min; 35 cycles of 94 °C for 1 min, then 57 °C for 1 min and 72 °C for 1 min 30 s; and 72 °C for 10 min. After amplification the PCR product was purified and checked on 1 % agarose gel. Size of the PCR amplicon was estimated by comparison with 1 Kb Generuler DNA molecular weight Marker (Fermentas). Purified PCR product was sequenced using ABI PRISM BigDye Terminator V3.1 Cycle sequencing kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. Homology searches were performed using the web-based BLAST service provided by National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The sequences obtained for wild type and P-87 were deposited in GenBank. The sequences alignment of all closest hits was performed using ClustalW [19] and phylogenetic analysis was performed using MEGA 5 software. The comparison of sequence was carried out to find out the variation in the sequence. The evolutionary history was inferred using UPGMA method [20]. The evolutionary distances were computed using Maximum Composite Likelihood method. The clustering of associated taxa in phylogenetic tree was performed using bootstrap test [21].

Sequencing of *rpIF* Gene

The bacterial genomic DNA of wild type and P-87 was extracted using ChargeSwitch® gDNA Mini Bacteria Kit (Life Technologies, USA) as per the manufacturer's instructions. For PCR amplification *rplF* gene specific primers were designed using Primer-BLAST tool (www.ncbi.nlm.nih.gov/tools/primer-blast) and reference sequence for *rplF* gene (Accession no. CP0004891). The sequence of primers was as following: rpIF-R (5′TCA TCCGTGAACTGGCCC3′) and rpIF-R (5′CCGAGAT CTTCCGCAACTTG3′). The PCR reaction mixture contained 100–200 ng of genomic DNA, 1X PCR buffer, 2.5 mM of $MgCl_2$, 0.1 mM of each dNTP, 0.2 mM of primer solution, 1.0 unit per reaction of Taq DNA polymerase and Nuclease free water to make total reaction volume of 25 µl. The PCR was performed at 94 °C for 5 min; 35 cycles of 94 °C for 45 s, then 54 °C for 45 s and 72 °C for 45 s; and 72 °C for 10 min. After amplification the PCR product was purified and checked on 1 % agarose gel. Size of the PCR amplicon was estimated by comparison with 1 Kb Generuler DNA molecular weight Marker (Fermentas). Purified PCR product was sequenced using

ABI PRISM BigDye Terminator V3.1 Cycle sequencing kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. Homology searches were performed using the web-based BLAST service provided by National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The sequences alignment of all closest hits was performed using ClustalW [19] and phylogenetic analysis was performed using MEGA 5 software. The comparison of sequence was carried out to detect the mutation at any position in the sequence.

Statistical Analysis

For analyzing differences between two groups, Student's *t* test was used based on PRISM-5 software. *P* values below 0.05 were considered statistically significant. The values in all graphs are an average of 3 trials. All error bars represent standard error of mean.

Accession Numbers of the Strains and Sequences Derived in this Study

The 16S rRNA gene sequences of mutant G-30 and P-87 was derived and deposited in GenBank (Accession number KF676725 and KF676726 respectively). Mutant G-30 and P-87 was deposited in microbial culture collection (MCC) of National Center for Cell Sciences (NCCS), Pune, India under accession number MCC2519 and MCC2520 respectively.

Results and Discussion

Mutagenesis and Rational Selection of Mutants

The wild type strain, *P. denitrificans* ATCC 19367 was found to produce considerably less amount of CoQ_{10} than the other few bacterial strains [7]. The improvement of *P. denitrificans* strain has not been reported till date for over production of CoQ_{10}. Our approach was to generate new mutant strains from *P. denitrificans* using physical and chemical mutagenesis followed by selection of antibiotic resistant strains as well as strains resistant to structural analogues of CoQ_{10}.

To begin with the strain, attempts were made to select a natural variant of *P. denitrificans* ATCC 19367 having resistance to higher concentration of cane molasses in the agar plate containing 13 % cane molasses. It was thought that the variant tolerating higher concentration of cane molasses may be able to assimilate higher quantity of carbon source during fermentation as mentioned in the earlier studies with *A. tumefaciens* [17]. In this process a single colony was found to be survived on the agar plate containing 13 % cane molasses. This variant was named as PdSr and used for further studies. The natural variant PdSr was further subjected to mutagenesis by γ irradiation using ^{60}Co as a source of γ rays having radiation frequency of 1.5 Kγ/h. The mutagenesis of *P. denitrificans* using γ irradiation has not been reported so far. In the initial pilot run, the wild type strain did not show any survival at 400 γ and above exposure dosed. Hence the irradiation dose was reduced in order to get the survival population having desired reduction in viability. For this purpose, the cells were exposed to different irradiation doses like 25, 50, 100 and 250 γ. Figure 1 shows the percent reduction in survival at different irradiation doses. Among different irradiation doses tried, irradiation of 250 γ showed maximum reduction in viability (99.99 %) with surviving mutant population. Hence the suspension exposed at 250 γ was used for selection of mutants. The selections of mutants were carried out based on the resistance to different antibiotics as well as structural analogues of CoQ_{10}. The mutants resistant to toxic structural analogues of product or antimetabolites are useful in strain improvement program. The structural analogue of CoQ_{10} namely menadione and idebenone were found to be toxic to the cells and therefore used as a marker to select the resistant mutants. The antibiotic resistant mutants were also selected in order to create resistant markers in the parental strains which may be further useful to carry out genetic recombination using protoplast fusion technology [22]. Initially antibiotic sensitivity test was performed on the wild type strain. Out of 30 antibiotics tested for sensitivity, the wild type strain was found to be sensitive towards 27 antibiotics having maximum sensitivity towards neomycin, vancomycin and gentamicin based on the lower MIC values using Hi Comb MIC test strips (Himedia). These 3 antibiotics were selected for generating resistant mutants using gradient plate technique. The γ irradiated suspension when plated on different antibiotics containing gradient plates, 93 no of vancomycin resistant mutant colonies growing at a concentration above MIC were obtained. Similarly 58 no of mutants resistant to idebenone and 69 no of mutants resistant to menadione were obtained. The efforts of selecting auxotrophic mutants using penicillin enrichment technique did not result in isolation of true auxotrophic mutant. Through shake flask screening of selected 220 mutants, vancomycin resistant mutant G-30 showed only 1.06-fold improvements in specific CoQ_{10} content than the wild type strain. The mutation and selection process is laborious hence developing high throughput methodologies for screening is essential. In order to achieve significant improvement in CoQ_{10} content, mutant G-30 was subjected to next round of mutagenesis using NTG. Around 96.09 % reductions in viability were observed with 400 μg/ml of

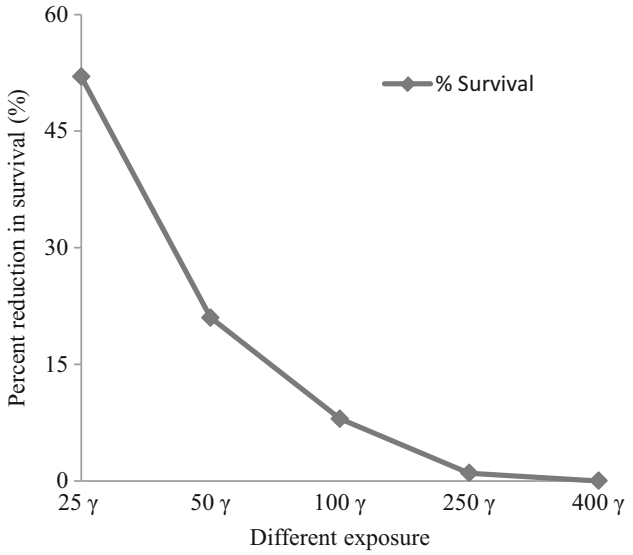

Fig. 1 Reduction in percent survival at different gamma irradiation doses on *P. denitrificans* ATCC 19367. The irradiation of 250 γ showed maximum reduction in cell viability

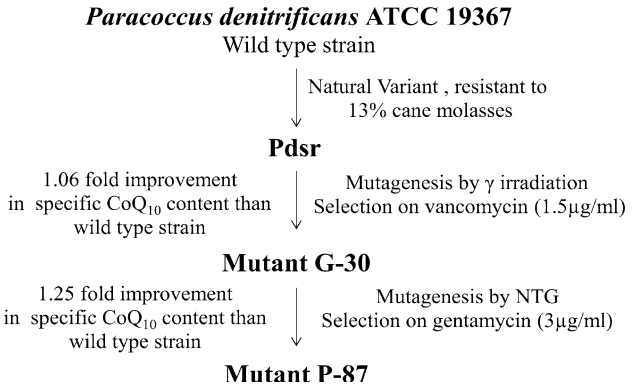

Fig. 2 Genealogy of mutant P-87 derived from *P. denitrificans* ATCC 19367 by sequential mutagenesis

NTG for 30 min of exposure. The rational selections of mutants were further carried out based on resistant to antibiotics and structural analogues of CoQ_{10} thereby 107 no of mutants resistant to gentamicin and 43 no of mutants resistant to menadione were obtained. The shake flask screening of these mutants resulted in selection of a gentamicin resistant mutant P-87 that showed 1.25-folds improvement in specific CoQ_{10} content than wild type strain which was highest among all mutants. The genealogy of P-87 is shown in Fig. 2. The antibiotic sensitivity pattern indicates that induced mutant G-30 acquired vancomycin (1.5 μg/ml) resistance where as mutant P-87 acquired resistance to gentamicin (3 μg/ml). The 1.25-folds improvement in specific CoQ_{10} content was achieved with gentamicin resistant mutant P-87. Hence it may be essential to investigate the cause of gentamicin resistance and its beneficial effect on CoQ_{10} improvement. The improvement in CoQ_{10} content of P-87 was not highly significant; hence further improvement is essential by means of mutagenesis and/or metabolic engineering.

Characterization of P-87 Based on Physiology and FAME Profile

Through the mutation and screening process, P-87 generated from wild type strain by sequential mutagenesis was found to accumulate high CoQ_{10} intracelleularly. This mutant was characterized in order to differentiate it from the wild type strain. The physiological characterization was carried out on the basis of carbohydrate assimilation test in order to select the best carbon source for designing the fermentation medium as well as to differentiate the strains based on carbohydrate assimilation. The P-87 showed differences in carbohydrate assimilation pattern than wild type strain with respect to dulcitol, inositol, inulin and mannose assimilation (Supplementary Table 2). The difference in the assimilation of various carbohydrates in P-87 indicates the possibility of having mutations on the metabolic pathway genes. Further differentiations of these strains were carried out using FAME profile. Based on the FAME profile, wild type and P-87 showed the presence of vaccenic acid (18:1w7c) as a major fatty acid contributing to 83 % of the total fatty acids (Supplementary Table 3). In addition to this, some minor new fatty acids were identified in P-87 namely trihydroxyundecanoic acid (11:0 3OH), linderic acid (12:1 3OH), myristic acid (14:00), iso pentadecanoic acid (15:0 iso), pentadecanoic acid (15:1 w5c), iso hexadecanoic acid (16:0 iso), eicosadienoic acid (20:2 w6,9c) and gondonic acid (20:1 w9c). The FAME profile of wild type and P-87 showed maximum similarity with *P. denitrificans* based on the Sherlock FAME database. The next closest match was obtained with *Rhodobacter spheroides*. The dendrogram was constructed using the FAME profile data of wild type and P-87 with their respective matches in the FAME library as shown in Fig. 3. The dendrogram revealed the differences between wild type and P-87 as they both are placed on the different clade having euclidian distance more than 2. The differences in the fatty acid profile as well as the physiological attributes indicated the novelty of mutant P-87.

Molecular Characterization Using AFLP and 16S rRNA Sequence

In order to differentiate the mutant strains (G-30 and P-87) from wild type strain at molecular level, the rapid DNA fingerprinting methods were attempted. The RAPD method

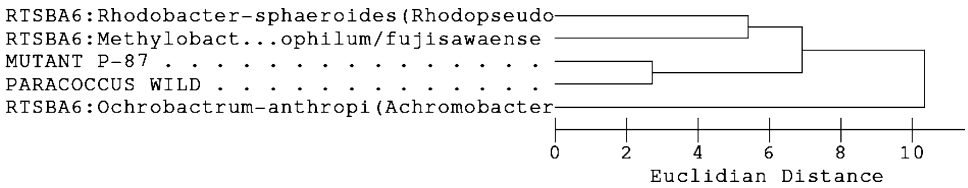

Fig. 3 The dendrogram of *P. denitrificans* ATCC 19367 (wild type) and mutant P-87 with their respective matches in the FAME library

using 20 different oligonucleotide primers did not work with these strains as no amplification was observed. Hence the DNA fingerprinting method by AFLP was followed. The AFLP analysis has been widely used to establish the genetic diversity among prokaryotic and eukaryotic species. It has emerged as a new powerful tool for genomic analysis. The ability to determine genetic variation among the species has been exploited in this study. Variation at the molecular level is directly related to the number of polymorphisms detected and their reproducibility. Five AFLP primer pairs were used to study the genetic diversity between wild type, G-30 and P-87. The reproducibility of AFLP fragments exceeded 99 % for each of the three strains based on the independent restriction-ligation reactions of each species that were amplified using five selective primer pairs (Supplementary Table 1). A total of 155 reproducible and clearly scorable bands, produced from five primer combinations, were assessed across the two strains. Out of these, 44 were monomorphic and 15 were polymorphic, resulted in 9.67 % overall polymorphism as shown in Table 1. The molecular weights of polymorphic fragments generated ranged from approximately 117 to 500 bp. Among the five primer pairs, the most informative primer combination was the EcoRI-FAM-0/MSeI-C pair, which produced eight different polymorphic bands (Supplementary Fig. 1). AFLP pattern analysis demonstrates that genotyping of wild type strain and two mutant strains could be accomplished on the basis of genomic variations with polymorphic band pattern using AFLP. The AFLP profiles using EcoRI/MseI AFLP primer kits were able to characterize intra-specific polymorphisms among wild type strain and two mutant strains. Cluster analysis of *P. denitrificans* strains was performed based on the AFLP data analysis as shown in Fig. 4. The phylogenetic analysis inferred using UPGMA method clearly indicated the genetic difference between wild type and mutant strains as all they all appeared on the different clades of the tree. The genetic distance between the strains was calculated as per the formula using band patterns and similarity coefficient [18]. The mean genetic distance between wild type and G-30 was found to be 0.149. The mean genetic distance between G-30 and P-87 was found to be 0.114. The mean genetic distance between wild type and P-87 was found to be 0.051. AFLP fingerprinting method has been used to detect genetic diversity of bacterial strains and to differentiate it from closely related strains. This method was found to be useful for detecting intra-specific polymorphism and is a powerful highly reproducible and discriminatory tool for revealing genetic relationships in bacterial populations [23]. The various strains of *Pseudomonas syringae* pv. *pisi* strains, representative of the plant pathogens seven races were clearly differentiated by AFLP fingerprinting. The AFLP profile provided a distinct profile for each of the strain [24]. Overall the AFLP method was found to be useful in discriminating the mutant strains G-30 and P-87 from the wild type strain at molecular level.

The 16S ribosomal RNA sequences of mutant G-30 and mutant P-87 were derived and deposited in NCBI GenBank (KF676725 and KF676726). The phylogenetic analysis was carried out based on the closest matching sequences (Supplementary Fig. 2). The sequence of G-30 and P-87 showed 100 % similarity with each other as well as with the reported sequence of *P. denitrificans* ATCC 19367. In the phylogenetic tree these strains appeared on the same clade and no mutations were detected on the sequence. Overall, 16S rRNA sequence showed close similarity between the two strains whereas AFLP could able to detect the minor mutation on other part of the gene sequence. Different approaches have been evolved in recent years for isolation and characterization of bacterial strains. The integrated approach using 16S rRNA phylogenetic framework, nucleotide signatures and in silico restriction enzyme digestion pattern was useful for identification of *Bacillus* spp. as well as *Clostridium* and *Pseudomonas* spp. [25–27]. Other than 16S rRNA sequence, other specific gene sequence has been used by researchers for phylogenetic analysis and identification of various bacterial species. Genomic analysis using quorum quenching enzymes have been carried out to identify versatile organism from mix cultures and metagenomes [28]. Genetic and functional diversity of *Stenotrophomonas* isolates from diverse effluent treatment plant have been evaluated using phylogenetic analysis of their 16S rRNA sequence, RAPD analysis as well as in silico signature and restriction enzyme digestion pattern [29]. The genetic and functional diversity of *Citrobacter* spp. isolated from different effluent treatment plant for their ability to degrade aromatic compound have been evaluated using aromatic ring-hydroxylating dioxygenase gene [30]. The *Stenotrophomonas* strain isolated from effluent treatment plant and having ability to

Table 1 AFLP profile obtained with wild type, mutant G-30 and mutant P-87 strain with overall polymorphism

Primer combination	Samples and total number of bands obtained after fragment analysis			Total number of fragments detected by each primer combination (a)	No of common bands	No of polymorphic bands (b)	% polymorphism obtained using primer combination (b/a) × 100 %
	Wild type	G-30	P-87	Total			
EcoR1-FAM-A/Msel-T	9	5	8	22	5	4	18.18 %
EcoR1-FAM-A/Msel-G	6	5	6	17	6	1	5.88 %
EcoR1-FAM-0/Msel-A	6	7	7	20	6	1	5.00 %
EcoR1-FAM-0/Msel-C	21	15	23	59	15	8	13.55 %
EcoR1-FAM-0/Msel-T	12	12	13	37	12	1	2.70 %
Total	54	44	57	155	44	15	9.67 % overall polymorphism

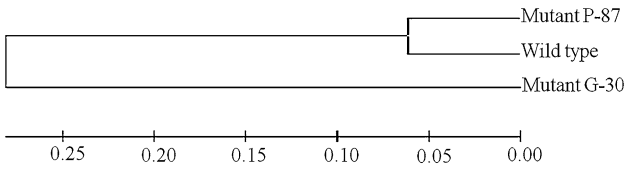

Fig. 4 Evolutionary relationships of taxa based on AFLP profile, showing relationship between *P. denitrificans* ATCC 19367, mutant G-30 and mutant P-87. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.71514138 is shown. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 [20, 21]

degrade various aromatic compound have been characterized based on the gene homologous dmp operon. It was observed that the sequenced DNA fragment exhibited close similarity to phenol hydroxylase gene from *Arthocater* spp. [31]. Further efforts were made to characterize mutant strain P-87 on the basis of *rpIF* gene that was expected to have been mutated causing gentamicin resistance.

Sequencing of *rpIF* Gene

The induced mutant P-87 showing improvement in CoQ_{10} production has acquired the resistance to gentamicin. The

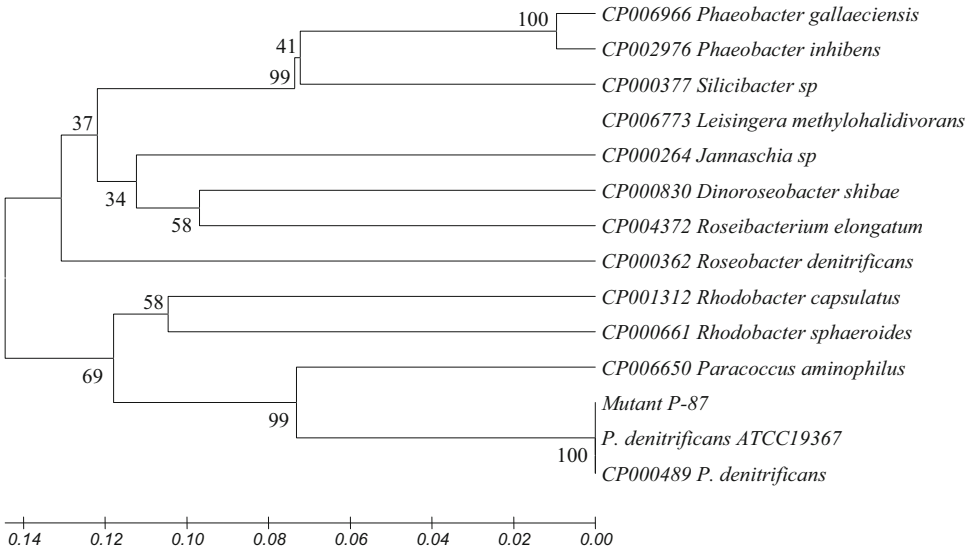

Fig. 5 Phylogenetic tree based on *rplF* gene sequences showing relationship between *P. denitrificans* ATCC 19367, mutant P-87 and closely related gene sequences. The evolutionary history was inferred using the UPGMA method [20]. The optimal tree with the sum of branch length = 1.20166792 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method [21] and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5

main acquired aminoglycoside resistance mechanism is enzymatic inactivation of the drug. Other mechanisms include target modification by mutations in ribosomal proteins or 16S rRNA, methylation of 16S rRNA, efflux, and diminished uptake impairment [32]. It was reported that, the mutations conferring resistant to ribosome targeting antibiotics helped in improving the productivity of secondary metabolites. The possible mechanism leading to overproduction may be the over expression of genes involved in ribosomal proteins and various enzymes in the biosynthetic pathway [33]. One of the reported causes of gentamicin resistance is conferred to the mutations on the ribosomal protein L6 gene (*rplF*), hence efforts were made to sequence *rplF* gene in wild type and P-87 for detecting the mutations. The *rplF* partial sequences of wild type and P-87 showed 100 % similarity through ClustalW alignment. Hence no mutations were detected. The phylogenetic analysis of *rplF* gene sequence from wild type and P-87 along with their closest matching hits was carried out and the phylogenetic tree is shown in Fig. 5. The wild type and P-87 were placed on the similar clade and it showed closest similarity with *Paracoccus aminophilus*. As no mutations were detected on the *rplF* partial gene sequence of P-87, the gentamicin resistance could not be correlated with the *rplF* genes. Hence investigating the cause of gentamicin resistance at molecular level and its possible role in CoQ_{10} overproduction may be essential.

Conclusions

Paracoccus denitrificans a biochemically versatile organism is known to produce CoQ_{10} as a major quinone. Due to its diverse metabolic activity it was thought to be an ideal organism to be utilized for CoQ_{10} fermentation, if the yields are improved. Therefore the efforts were made to improve the type strain of *P. denitrificans* ATCC 19367 for improved production of CoQ_{10}. With the first report, a novel mutant strain P-87 resistant to gentamicin (3 μg/ml) was generated by iterative rounds of mutagenesis and selections with 1.25-fold improvement in specific CoQ_{10} content than wild type strain. Among different rational selection methods tried like, resistance to antibiotics as well as toxic structural analogues, the gentamicin resistance helped generating improved mutant strain. The mutant P-87 was differentiated from the wild type train using carbohydrate assimilation and FAME profile. The DNA fingerprinting method of AFLP was useful in differentiating the mutant strain from the wild type strain. Hence primers used in the AFLP study may be useful in differentiating the strains and species of genus *Paracoccus*. The gentamicin resistance in P-87 could not be correlated with *rplF* gene as no mutations were observed on this gene. Hence investigating the cause of gentamicin resistance and correlating this phenotypic marker with improved CoQ_{10} production in P-87 is essential.

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References

1. Jeya M, Moon HJ, Lee JL, Kim IW, Lee JK (2010) Current state of coenzyme Q10 production and its application. Appl Microbiol Biotechnol 85:1653–1663. doi:10.1007/s00253-009-2380-2
2. Choi JH, Ryu YW, Seo JH (2005) Biotechnological production and applications of coenzyme Q10. Appl Microbiol Biotechnol 68:9–15. doi:10.1007/s00253-005-1946-x
3. Littarru G (2007) Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. Mol Biotechnol 37:31. doi:10.1007/s12033-007-0052-y
4. Schmelzer C, Okun JG, Haas D, Higuchi K, Sawashita J, Mori M, Doring F (2010) The reduce form of coenzyme Q10 mediates distinct effects on cholesterol metabolism at the transcriptional and metabolic level in SAMP1 mice. IUBMB Life 62:812–818. doi:10.1002/iub.388
5. Inui M, Ooe M, Fuji K, Matsunaka H, Yoshida M, Ichihashi M (2008) Mechanisms of inhibitory effects of coQ10 on UVB-induced wrinkle formation in vitro and in vivo. BioFactors 32:237–243. doi:10.1002/biof.5520320128
6. Ravada SR, Emani LR, Garaga MR, Meka B, Golakoti T (2009) Synthesis of coenzyme Q10. Am J Infect Dis 5:83–89. doi:10.3844/ajidsp.2009.83.89
7. Yoshida H, Kotani Y, Ochiai K, Araki K (1998) Production of ubiquinone-10 using bacteria. J Gen Appl Microbiol 44:19–26. doi:10.2323/jgam.44.19
8. Cluis CP, Pinel D, Martin VJJ (2012) The production of coenzyme Q10 in microorganisms. In: Wang X (ed) Reprogramming microbial metabolic pathways. Springer, New York. doi:10.1007/978-94-007-5055-5_15
9. Rao RS, Jyothi CP, Prakasham RS, Rao CS, Sarma PN, Rao LV (2006) Strain improvement of *Candida tropicalis* for the production of xylitol: biochemical and physiological characterization of wild-type and mutant strain CT-OMV5. J Microbiol 44:113–120
10. Ranadive P, Mehta A, George S (2011) Strain improvement of *Sporidiobolus johnsonii* ATCC 20490 for biotechnological production of Coenzyme Q10. Int J Chem Eng Appl 2:216–220. doi:10.7763/IJCEA.2011.V2.106
11. Ranadive P, Mehta A, Chavan Y, Marx A, George S (2014) Morphological and molecular differentiation of *Sporidiobolus johnsonii* ATCC 20490 and its coenzyme Q10 overproducing mutant strain UF16. Indian J Microbiol 54:343–357. doi:10.1007/s12088-014-0466-8
12. Cardoso PG, Queiroz MV, Pereira OL, Araujo EF (2007) Morphological and molecular differentiation of the pectinase producing fungi *Penicillium expansum* and *Penicillium*

griseoroseum. Braz J Microbiol 38:71–77. doi:10.1590/S1517-83822007000100015
13. Aparajita S, Rout GR (2010) Molecular analysis of *Albizia* species using AFLP markers for conservation strategies. J Genet 89:95–99 28A
14. Harrigan WF (1998) Laboratory methods in food microbiology, 3rd edn. Academic Press, San Diego
15. Dixson DD, Boddy CN, Doyle RP (2011) Reinvestigation of coenzyme Q10 isolation from *Sporidiobolus johnsonii*. Chem Biodivers 8:1033–1051. doi:10.1002/cbdv.201000278
16. Ha SJ, Kim SY, Seo JH, Lee KM, Lee JK (2007) Controlling the sucrose concentration increases coenzyme Q10 production in fed-batch culture of *Agrobacterium tumefaciens*. Appl Microbiol Biotechnol 76:109–116. doi:10.1007/s00253-007-0995-8
17. Tokdar P, Wani A, Kumar P, Ranadive P, George S (2013) Process and strain development for reduction of broth viscosity with improved yield in coenzyme Q10 fermentation by *Agrobacterium tumefaciens* ATCC 4452. Ferment Technol. doi:10.4172/2167-7972.1000110
18. Nei M, Li W (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci 76:5269–5273. doi:10.1073/pnas.76.10.5269
19. Larkin MA, Blackshields G, Brown NP et al (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948. doi:10.1093/bioinformatics/btm404
20. Sneath PHA, Sokal RR (1973) Numerical Taxonomy. Freeman, San Francisco Unweighted Pair Group Method with Arithmetic Mean; 230–234
21. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739. doi:10.1093/molbev/msr121
22. Gong J, Zheng H, Wu Z, Chen T, Zhao X (2009) Genome shuffling: progress and applications for phenotype improvement. Biotechnol Adv 27:996–1005. doi:10.1016/j.biotechadv.2009.05.016
23. Rosaa GL, Carolisa ED, Salia M, Papacchinib M, Riccardib C, Mansic A, Pabac E, Alquatid C, Bestettid G, Muscilloa M (2006) Genetic diversity of bacterial strains isolated from soils, contaminated with polycyclic aromatic hydrocarbons, by 16S rRNA gene sequencing and amplified fragment length polymorphism fingerprinting. Microbiol Res 161:150–157. doi:10.1016/j.micres.2005.07.006
24. Cirvilleri G, Scuderi G, Catara V, Scortichini M (2007) Typing of *Pseudomonas syringae* pv. *pisi* strains by fluorescent AFLP fingerprinting. J Plant Pathol 89:421–425
25. Porwal S, Lal S, Cheema S, Kalia VC (2009) Phylogeny in aid of the present and novel microbial lineages: diversity in *Bacillus*. PLoS ONE 4:e4438. doi:10.1371/journal.pone.0004438
26. Kalia VC, Mukherjee T, Bhushan A, Joshi J, Shnkar P, Huma N (2011) Analysis of the unexplored features of rrs (16S rDNA) of the genus *Clostridium*. BMC Genom 12:18. doi:10.1186/1471-2164-12-18
27. Bhushan A, Joshi J, Shankar P, Kushwah J, Raju SC, Purohit HJ, Kalia VC (2013) Development of genomic tools for the identification of certain *Pseudomonas* up to species level. Indian J Microbiol 53:253–263. doi:10.1007/s12088-013-0412-1
28. Kalia VC, Raju SC, Purohit HJ (2011) Genomic analysis reveals versatile organisms for quorum quenching enzymes: acyl-homoserine lactone-acylase and lactonase. Open Microbiol J 5:1–13. doi:10.2174/1874285801105010001
29. Verma V, Raju SC, Kapley A, Kalia VC, Daginawala HF, Purohit HJ (2010) Evaluation of genetic and functional diversity of *Stenotrophomonas* isolates from diverse effluent treatment plants. Bioresour Technol 101:7744–7753. doi:10.1016/j.biortech.2010.05.014
30. Selvakumaran S, Kapley A, Kashyap SM, Daginawala HF, Kalia VC, Purohit HJ (2011) Diversity of aromatic ring-hydroxylating dioxygenase gene in *Citrobacter*. Bioresour Technol 102:4600–4609. doi:10.1016/j.biortech.2011.01.011
31. Verma V, Raju SC, Kapley A, Kalia VC, Kanade GS, Gaginawala HF, Purohit HJ (2011) Degradative potential of *Stenotrophomonas* strain HP383 having genes homologous to dmp operon. Bioresour Technol 102:3227–3233. doi:10.1016/j.biortech.2010.11.016
32. Aslangul E, Massias L, Meulemans A, Chau F, Andremont A, Courvalin P, Fantin B, Ruimy R (2006) Acquired gentamicin resistance by permeability impairment in *Enterococcus faecalis*. Antimicrob Agents Chemother 50:3615–3621. doi:10.1128/AAC.00390-06
33. Tamehiro N, Hosaka T, Xu J, Hu H, Otake N, Ochi K (2003) Innovative approach for improvement of an antibiotic overproducing industrial strain of *Streptomyces albus*. Appl Environ Microbiol 69:6412–6417. doi:10.1128/AEM.69.11.6412-6417.2003