A Closer Look on the Polyhydroxybutyrate- (PHB-) Negative Phenotype of *Ralstonia eutropha* PHB-4

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Abstract

The undefined poly(3-hydroxybutyrate)- (PHB-) negative mutant *R. eutropha* PHB-4 was generated in 1970 by 1-nitroso-3-nitro-1-methylguanidine (NMG) treatment. Although being scientific relevant, its genotype remained unknown since its isolation except a recent first investigation. In this study, the mutation causing the PHA-negative phenotype of *R. eutropha* PHB-4 was confirmed independently: sequence analysis of the *phaCAB* operon identified a G320A mutation in *phaC*, yielding a stop codon, leading to a massively truncated PhaC protein of 106 amino acids (AS) in *R. eutropha* PHB-4 instead of 589 AS in the wild type. No other mutations were observed within the *phaCAB* operon. As further mutations probably occurred in the genome of mutant PHB-4 potentially causing secondary effects on the cells' metabolism, the main focus of the study was to perform a 2D PAGE-based proteome analysis in order to identify differences in the proteomes of the wild type and mutant PHB-4. A total of 20 differentially expressed proteins were identified which provide valuable insights in the metabolomic changes of mutant PHB-4. Besides excretion of pyruvate, mutant PHB-4 encounters the accumulation of intermediates such as pyruvate and acetyl-CoA by enhanced expression of the observed protein species: (i) ThjH supports biosynthesis of cofactor TPP and thereby reinforces the 2-oxoacid dehydrogenase complexes as PDHC, ADHC and OGDHC in order to convert pyruvate at a higher rate and the (ii) 3-isopropylmalate dehydrogenase LeuB3 apparently directs pyruvate to synthesis of several amino acids. Different (iii) acylCoA-transferases enable transfer reactions between organic acid intermediates, and (iv) citrate lyase CitE4 regenerates oxaloacetate from citrate for conversion with acetyl-CoA in the TCC in an anaplerotic reaction. Substantial amounts of reduction equivalents generated in the TCC are countered by (v) synthesis of more ubiquinones due to enhanced synthesis of MenG2 and MenG3, thereby improving the respiratory chain which accepts electrons from NADH and succinate.

Introduction

*Ralstonia eutropha* H16 is a Gram-negative, rod-shaped and facultative chemolithoautotrophic hydrogen-oxidizing bacterium belonging to the *β*-proteobacteria. *R. eutropha* appears as a ubiquitous inhabitant of soil and freshwater habitats. Although respiration plays the major role in energy generation, this bacterium is well adapted to transient anoxia [1]. *R. eutropha* is able to oxidize molecular H₂ and various organic compounds. Two hydrogenases catalyze the oxidation of H₂ during lithoautotrophic growth: one membrane bound hydrogenase transfers electrons into the electron transport chain, and one cytosolic hydrogenase generates reducing power (NADH) for CO₂ fixation. Autotrophic CO₂ fixation is mediated by the Calvin-Benson-Bassham cycle [2]. Furthermore, *R. eutropha* is in addition able to use various organic carbon and energy sources for heterotrophic growth including TCA cycle intermediates, sugar acids like gluconic acid, fatty acids or other acids and amino acids. Notably, the capability of H16 to metabolize sugars is restricted to fructose and N-acetylg glucosamine [3].

Besides playing a key role in investigating hydrogen-based chemolithoautotrophy, *R. eutropha* serves as model organism of polyhydroxyalkanoate (PHA) metabolism since more than 50 years [4,5]. PHAs are accumulated by a large number of prokaryotes and serve as intracellular storage compounds for carbon and energy and can be used for various applications in industry and medicine due to their thermoplastic properties and biodegradability [6]. The most frequently found polymer of this class is poly(3-hydroxybutyrate) (PHB), which was firstly described by Lemoigne [7] in *Bacillus megaterium*.

PHAs are synthesized in batch or fed-batch cultures under unbalanced growth conditions if a carbon source is present in excess and if another macroelement (N, O, P, S) is depleted at the same time [8,9]. PHAs may represent the major cell constituent excess and if another macroelement (N, O, P, S) is depleted at the same time [8,9]. PHAs may represent the major cell constituent contributing up to 90% of the cell dry weight [10]. Although *R. eutropha* H16 is able to synthesize different PHAs of short carbon chain length [11], PHB is usually the predominant PHA in this bacterium [12,13].

In *R. eutropha* synthesis of PHB proceeds in three steps catalyzed by the enzymes β-ketothiolase (PhaA), acetoacetyl-CoA reductase...
(PhaB) and PHA synthase (PhaC) (14–17). The genes for these three enzymes are located in the PHA-operon phaCAB [19–20]. Whereas PhaC is essential for PHA biosynthesis in R. eutropha H16, PhaA and PhaB can be replaced by isoenzymes [21,22]. A second PHA synthase gene was identified within the R. eutropha H16 genome sequence project [23], which putatively encodes an additional PHA synthase in this bacterium. However, this phaC2 annotated gene is obviously not transcribed in H16 [24]. In the first step, two molecules acetyl-CoA are condensed to acetoacetyl-CoA by a β-ketothiolase (PhaA) [14,17]. The second reaction is catalyzed by the NADPH-dependent acetoacetyl-CoA reductase (PhaB) yielding (R)-3-hydroxybutyryl-CoA (3HB-CoA) [15]. The PHA synthase (PhaC) finally polymerizes the 3HB moieties of 3HB-CoA to PHB [16,25]. The PHA synthase (PhaC) is regarded as the key enzyme of PHA synthesis and many phaC genes have been cloned and characterized [26–28]. According to their molecular constitution and their substrate specificity PHA synthases are divided into four classes: Class I enzymes form short-chain-length (SCL) PHAs and their substrate specificity PHA synthases are divided into four groups [26–28]. According to their molecular constitution the rRNA homology group I and accept monomers of medium- and long-chain-length (MCL) comprising six to fourteen carbon atoms (C6–C14) [28,32]. In contrast to the above mentioned enzymes, synthases of class III consist of two different subunits (PhaC + PhaE) and convert short-chain-length (SCL) monomers; they are found in purple sulfur bacteria, sulfate-reducing bacteria and others [33]. Likewise composed of two different subunits (PhaC + PhaK), PHA synthases of class IV are represented by enzymes of Bacillus sp. and prefer short-chain-length (SCL) monomers [34]. PHA synthases exhibit generally a broad substrate specificity, and also PhaC of R. eutropha catalyzes the synthesis of a large number of PHAs composed of diverse SCL hydroxyalkanoic acids as well as polythioesters (PTEs) consisting of mercaptoalkanoic acids [35,5]. At the beginning of the 1960ties R. eutropha and derived mutants attracted interest as producer of single cell protein (SCP) for animal nutrition from CO2 and H2 [36]. Similar attempts were conducted with diverse other microorganisms applying various carbon sources [37], but this program was finally stopped due to economic reasons as SCP was not able to compete with classical feedstock and because cells harboured too high concentrations of disturbing RNA. During these investigations, PHB-negative mutants were isolated [38,39] which became important in research, later. Originally these mutants were isolated because the animals’ digestive tracts are unable to degrade PHB which has therefore no nutritional value. The most prominent representative of the PHB-negative mutants became R. eutropha PHB-4. This strain and similar mutants derived from 1-nitroso-3-nitro-1-methylguanidine (NMG)-induced PHB-negative mutant R. eutropha PHB-4 ([38], DSM 541, SK-No.: 7387) were used in this study. Cultivations in liquid media were done in Erlemeyer or Klett flasks with baffles on a rotary shaker at an agitation of 125 r.p.m. Cells of R. eutropha for proteomic studies were grown in 2 L Klett flasks equipped with baffles at 30°C in 300 ml mineral salts medium (MSM) [41]. These cultivations were done in triplicate to allow generation of 2D PAGE gel replicates from three independent experiments for a subsequent computer based proteome analysis. Media contained sodium glutonate (1.0%, wt/vol) as carbon source and low amounts of NH4Cl (0.05%, wt/vol) as nitrogen source to allow PHB synthesis in the wild type H16. When cells had reached the exponential growth phase after 10 hours of cultivation, the first sample was withdrawn and, after in total 26 hours of cultivation, the second sample was withdrawn in the stationary growth phase. The samples of the wild type H16 and mutant PHB-4 were subsequently subjected to proteome analyses.

**Escherichia coli.** Top10 (Invitrogen) was cultivated in Luria-Bertani (LB) medium [42] at 37°C. Solid media contained 1.8% (wt/vol) agar. If required, 75 µg Ampicillin (Ap) ml−1 was added for E. coli. Isolation, manipulation and transfer of DNA

Isolation of genomic DNA of R. eutropha was done according to Marmur [43]. Plasmid DNA was isolated by the method of Birnboim and Doly [44]. DNA manipulations and other standard molecular biology techniques were performed according to Sambrook et al. [42]. Amplifications of genomic DNA were made using Herculase II Fusion DNA polymerase (Agilent Technologies) in an Omnimed HBT1R3CM DNA thermocycler (Hybaid) employing oligonucleotide primers mentioned below. Obtained sequences were analyzed using Chromas software (version 1.45, Technelysium Pty Ltd), Genamics Expression software (version 1.100 [http://genamics.com/ expression/index.html]), BLAST on-line service available on NCBI [National Center for Biotechnology Information [http://blast.ncbi.nlm.nih.gov/Blast.cgi]], and BioEdit [45]. Competent cells of E. coli were prepared and transformed by the CaCl2 procedure as described by Hanahan [46].

**Sequence analysis of the phaCAB operon of the PHB-negative mutant R. eutropha PHB-4**

For sequencing the genomic region of R. eutropha PHB-4 from 386 bp upstream of gene phaC to 123 bp downstream of gene phaB comprising the whole phaCAB operon, a 4860 bp DNA fragment was generated by PCR using Herculase II Fusion DNA Polymerase (Agilent Technologies) and primers phaCAB_fw: GCGGATGAAACAGGTTCCGGGTTGC and phaCAB_rv: GCCCTTGACGGCGCGAACGG applying genomic DNA of mutant R. eutropha PHB-4 as template. The purified PCR product (peqGOLD Gel Extraction Kit, peqlab) was ligated with...
vector pJET1.2/blunt and applied for transformation of competent E. coli TOP10 cells according to the CloneJET PCR Cloning Kit (Thermo Scientific). Recombinant plasmids were isolated from positive E. coli clones, and three independently obtained plasmids were subjected to DNA sequence analysis using the sequencing primers listed in Table 1 in order to generate overlapping sequences to ensure sufficient coverage.

DNA sequencing was carried out at the Sequence Laboratories Göttingen (Seqlab). Obtained sequences were assembled and analysed using Chromas software (version 1.45, Technelysium Pty Ltd). Sequence comparisons and alignments were performed using the BLAST online service available on NCBI (National Center for Biotechnology Information [http://blast.ncbi.nlm.nih.gov/Blast.cgi]), BioEdit [45] and ClustalW [47].

Preparation of protein samples for proteome analysis

To obtain crude extracts from cells which were cultivated in MSM, cells were harvested by centrifugation (15 min, 3,500 g, 4°C), and the resulting cell pellets were then treated with crack solution (8 M Urea, 2%, vol/vol, Triton X-114). The volume of crack solution corresponded to the volume of the centrifuged cell suspension, i.e. a pellet that resulted from 50 ml culture broth was resuspended in 50 ml crack solution. The mixture was then incubated for 1.5 h at room temperature on a gyratory shaker to break the cells. PHB and cell debris were removed from the crude extract by centrifugation (1 h, 60,000 to 70,000 g, 4°C). Proteins were subsequently extracted from the supernatant by phenol extraction as described before [48]. After aceton precipitation, the washed protein pellet was air-dried by incubation at room temperature to evaporate the acetone, and was then stored at −20°C.

Rehydration of proteins

An adequate volume of rehydration buffer A (Urea 9 M, CHAPS 4%, wt/vol, DTT 100 mM, H2Odest. ad 10 ml) in dependency on pellet size was added to the dry protein pellets, and the mixture was then incubated at room temperature for 2 h. To ensure effective rehydration, the samples were stirred several times during this period. Protein solutions were then transferred to 1.5 ml plastic tubes and centrifuged (5 min, 11,000 g). The protein concentration in the supernatants was subsequently measured.

Determination of protein concentration

To reduce the disturbing influence of DTT and Urea [49], which are part of rehydration buffer A, only 5 μl of highly concentrated protein solution were mixed with a volume of 5 ml of Bradford reagent (70 mg Serva Blue G, 50 ml 96% vol/vol, ethanol, 100 mg 85%, vol/vol, phosphoric acid, H2Odest. ad 1,000 ml) according to Bradford [50]. After 10 min incubation in the absence of light, the absorption at 595 nm against the reagent blank value was measured. A calibration was done with bovine serum albumin (BSA) in the range from 1 to 1,000 μg.

2D PAGE: first-dimension isoelectric focusing (IEF)

An aliquot of protein solution that contained 1.5 mg protein was filled up to 200 μl with rehydration buffer A and was mixed with 150 μl rehydration buffer B (2.5 ml rehydration buffer A, 125 μl amphotolyt solution pH 3–10 [Serva], 125 μl Triton X-100, trace amount of Bromophenol Blue). The IEF strips (pH 5–8, 11 cm, BioRad) were passively rehydrated over night at room temperature with 350 μl of prepared protein solutions while overlaid with mineral oil. After rehydration of the strips with the protein solutions, strips were focussed in a focussing tray (while overlaid with mineral oil) by a series of voltage increases: 250 V (1 h), 500 V (1 h), 1,000 V (1 h), 6,000 V (18 h) and 500 V (up to 99 h) at 20°C to a total value of at least 100 kVh.

2D PAGE: second dimension and gel staining

The equilibration of IEF strips, 2D gel-run in a DODECA-cell (BioRad) as well as staining and destaining procedures were done exactly as described in Raberg et al. [48].

Software based analysis of 2D gel images

Images from 2D PAGE were analysed separately for three independent biological experiments using the DECODON Delta 2D software (Decodon GmbH, Greifswald) due to method immanent variations in 2D PAGE gels based on different cultivations, and one representative analysis is presented here in

Table 1. Oligonucleotide primers used for sequencing of the phaCAB operon from R. eutropha PHB-4.

| Primer  | Description                  | Location   |
|---------|------------------------------|------------|
| Seq1_fw| GCAAGCATAGCCATGGCGGTCTCC     | Upstream phaC |
| Seq2_rv| GGAGACGCGATCGCTAGTGCTGC     | Upstream phaC |
| Seq3_rv| CCAAGCGCCGAGTTGTCGCC        | Upstream phaC |
| Seq4_fw| CTTTGACCAGGCTGGCCGATGCC     | Within phaC |
| Seq5_fw| CCAAGACGCCGCCAAGCCTCC       | Within phaC |
| Seq6_fw| CCAGCTGGCAGTCACAGCGGCG      | Within phaC |
| Seq7_rv| CGTCTGCTACCAGCGTCTGG        | Within phaC |
| Seq8_rv| CCGCATGGTGCCGCGGGC          | Within phaC |
| Seq9_rv| CGGAGGACCGGCTGGGCGGATGAGTACATGG | Within phaA |
| Seq10_rv| GGCTCTGGGACGTGTAACCATGACC | Within phaA |
| Seq11_fw| GCTCGCAGAACAAGGCGGAAGC     | Within phaA |
| Seq12_rv| GGTGGTCTGAGCCGCGCCAAGG     | Within phaA |
| Seq13_rv| GCCATGCGTGTACGCGGACAGCGC | Within phaB |
| Seq14_rv| GCTGACCTGGGACCTGACCAAGAGCC | Within phaB |

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Proteomic View on Mutant R. eutropha PHB-4

As R. eutropha PHB-4 is an undefined chemically induced mutant, further mutations in addition to the identified G320A mutation in phaC probably occurred in the genome of strain PHB-4 which are not necessarily related to the PHB-negative phenotype. On the other side, many mutations can cause secondary effects on a cells’ metabolism. To address such unpredictable effects, a 2D PAGE based proteome analysis was conducted in order to identify differences in the proteomes of the wild type H16 and the mutant PHB-4 in the exponential and in the stationary growth phase, respectively.

Differentially expressed proteins were identified applying the DECODON Delta 2D software (Decodon GmbH, Greifswald), protein spots were excised from 2D gels and subjected to MALDI-TOF analysis. Doing so, protein species from a total of 20 spots were identified. These spots were marked and numbered in the dual views of the average fusions of the replicate 2D gel groups (Fig. 2), the corresponding proteins were listed (table 2) and densitometrically quantified according to the respective relative spot volume in the gels (Fig. 3). While 17 proteins were increasingly expressed in mutant PHB-4, four protein spots (#17–20) showed larger amounts in gels of the wild type H16 and consisted of isoforms of the same protein (PhaP1) with same Mr but differing pl’s. Phasin PhaP1 represents the quantitatively predominant phasin protein of seven homologues found in R. eutropha H16 [53–55], shielding the surface of PHA granules from the cytosol [56].

Among the proteins being increasingly expressed in strain PHB-4, five isoforms of an AcylCoA-transferase were observed. Expression of these protein species interestingly originated from two different gene loci, namely H16_A2386 (#1, 5, 7) and H16_B2114 (#8, 9). Generally, CoA-transferases catalyse reversible transfer reactions of coenzyme A groups from CoA-thioesters to free acids [57]. Furthermore, the ω- and β-subunits of the E1 component of the acetoacet dehydrogenase complex [AcoA, AcoB] showed higher synthesis in R. eutropha PHB-4 as indicated by spots 2, 3 and 4. The acetoacet dehydrogenase complex (ADHC) shares a related structure with the family of 2-oxoacid dehydrogenase complexes. These multienzyme complexes consist of three principal enzyme components: a substrate-specific decarboxylase/dehydrogenase (E1), a complex-specific dihydrolipoamide
acetyltransferase (E2), and a nonspecific dihydrolipoamide dehydrogenase (E3) [58,59]. In *R. eutropha*, acetoin is metabolized by the ADHC to acetaldehyde and acetyl-CoA [60,61]. Spot 11 consisted mainly of ThiJ and was likely stronger expressed in the mutant. ThiJ and its close orthologues are kinases involved in the biosynthesis of thiamine pyrophosphate (TPP) [62]. TPP is an essential cofactor of the E1 component of the 2-oxoacid dehydrogenase complexes [63,64]. Additionally, the citrate lyase CitE4 (β subunit, #10) showed a strong expression, especially in the exponential growth phase of PHB-4. This enzyme influences the tricarboxylic acid cycle (TCC) by an anaplerotic reaction as this enzyme catalyses the conversion of citrate to acetate and oxaloacetate, and vice versa [65]. The succinyl-CoA transferase (α subunit) being apparent in two spots (#12 and 14) was another protein connected to the TCC that was identified. The succinyl-CoA transferase catalyzes the transfer of coenzyme A from succinyl-CoA to acetoacetate [66]. Further proteins which showed higher expression levels were the 3-isopropylmalate dehydrogenase LeuB3 (#6), a triosephosphate isomerase (Tim, #11), the demethylmenaquinone methyltransferases MenG2 (#12–14) and MenG3 (#16) and SspA, a stringent starvation protein A (#15).

**Discussion**

Sequence analysis of the phaCAB operon of the PHB-negative mutant *R. eutropha* PHB-4

The observed G320A point mutation introduced a stop codon in *phaC* of *R. eutropha* PHB-4 (Fig. 1), leading to a significantly truncated PHA synthase PhaC. Consequently, a complete loss of the catalytic activity was principally expected for the remaining PhaC protein as already shown in previous studies [20]. Supporting this assumption, Rehm presented in 2003 [67] a primary structure analysis of PhaC.

![Figure 1. Schematic presentation of the phaCAB operon of *R. eutropha* PHB-4.](image1)

![Figure 2. Changes in the proteomes of the PHA-negative mutant *R. eutropha* PHB-4 compared to the wild type *R. eutropha* H16.](image2)
explain the PHB-negative phenotype of active site Cys-319, the conserved His-508 and the Asp-480, clearly.

eutropha PHA synthase, forming a catalytic triade composed of the enzyme, although several conserved blocks were not tolerated and likewise lead to an inactive enzyme. These data together with the proposed protein model of the PHA synthase. Therefore, this N-terminal region is not essential for the enzymatic activity of the PHA synthase.

In contrast, deletions at the C-terminus did abolish PHA synthase activity, suggesting that the C-terminus is essential for enzymatic activity in R. eutropha. Further deletions in PhaC within or between conserved blocks were not tolerated and likewise lead to an inactive enzyme, although several SmaI restriction sites [69], site-specific deletions [69], and PCR-mediated random mutagenesis [70]. The corresponding data indicated that mutations in the highly variable N-terminus (the first 100 amino acid residues) or even the deletion of the entire first 100 N-terminal amino acid residues did not inactivate the enzyme. Therefore, this N-terminal region is not essential for the enzymatic activity of the PHA synthase.

from R. eutropha summarizing the effect of various site specific mutants. These mutations were performed by Rehm and coworkers in the Steinbuchel laboratory by themselves or other laboratories and comprised insertions of Smal restriction sites [68], site-specific deletions [69], and PCR-mediated random mutagenesis [70]. The corresponding data indicated that mutations in the highly variable N-terminus (the first 100 amino acid residues) or even the deletion of the entire first 100 N-terminal amino acid residues did not inactivate the enzyme. Therefore, this N-terminal region is not essential for the enzymatic activity of the PHA synthase.

Proteome analysis of the PHB-negative mutant R. eutropha PHB-4 in comparison to the wild type R. eutropha H16

Cells of R. eutropha, which were subjected to proteome analyses, were cultivated in MSM under conditions promoting PHB synthesis by providing a carbon source (gluconate) in excess but limiting the nitrogen source. When cells became stationary due to nitrogen limitation, cells of R. eutropha PHB-4 were faced with an excess of intermediates and products of the KDPG pathway which metabolizes the gluconate leading to pyruvate. Part of the pyruvate is subsequently converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDHC). In contrast to the wild type H16, mutant PHB-4 is not able to direct these amounts of acetyl-CoA towards synthesis and accumulation of PHB. Under these conditions, mutant PHB-4 excretes large amounts of pyruvate. This behavior is a general characteristic of all PHB-negative mutants investigated so far; pyruvate excretion was observed when spontaneous and chemically or transposon-induced mutants were exposed to conditions which are permissive for the accumulation of PHB in the wild type cells [71–73]. At comparable cultivation conditions to those in this study, i.e. applying MSM with excess gluconate but limited ammonium provision, the PHB-negative mutant excreted up to 40 mM pyruvate in the stationary growth phase leading to a decrease of the pH value in the medium to pH 5 [73].

The proteome analysis revealed several metabolic adaptations of mutant PHB-4 to this situation. Figure 4 gives a schematic overview of the relevant parts of the metabolism of R. eutropha H16 and mutant PHB-4. One protein, which was synthesized in larger amounts in the mutant, was the kinase ThiJ, which is involved in biosynthesis of TPP, the essential cofactor of the 2-oxoacid dehydrogenase complexes [63,64]. 2-Oxo-acid dehydrogenase complexes convert 2-oxo acids to the corresponding acyl-CoA derivatives and produce NADH and CO₂ in an irreversible reaction. Five members of this family are known at present, the

Table 2. Differentially expressed proteins obtained by proteome analysis.

| Spot # | Rank | Annotation                                      | Accession No. |
|--------|------|------------------------------------------------|--------------|
| 1      | 1    | Predicted acyl-CoA transferase/carnitine dehydratase | H16_A2386    |
| 2      | 1    | AcGB acetoxy dehydrogenase E1 component beta-subunit (EC 1.2.4.1) | H16_B0145    |
| 3      | 1    | AcGB acetoxy dehydrogenase E1 component alpha-subunit (EC 1.2.4.1) | H16_B0144    |
| 4      | 1    | AcGB acetoxy dehydrogenase E1 component alpha-subunit (EC 1.2.4.1) | H16_B0144    |
| 5      | 1    | Predicted acyl-CoA transferase/carnitine dehydratase | H16_A2386    |
| 6      | 1    | LeuB3 3-Isopropylmalate dehydrogenase (EC 1.1.1.85) | H16_A2619     |
| 7      | 1    | Predicted acyl-CoA transferase/carnitine dehydratase | H16_A2386    |
| 8      | 1    | Predicted acyl-CoA transferase/carnitine dehydratase | H16_B2114    |
| 9      | 1    | Predicted acyl-CoA transferase/carnitine dehydratase | H16_B2114    |
| 10     | 1    | CitE4 citrate lyase beta subunit (EC 4.1.3.6) | H16_B2113     |
| 11     | 1    | ThpI putative intracellular protease/amidase/DJ-1/Pfli family | H16_A0394    |
| 12     | 2    | Tim triose phosphate isomerase (EC 5.3.1.1) | H16_A1047     |
| 13     | 1    | Succinyl-CoA-3-ketoacid-coenzyme A transferase subunit A (EC 2.8.3.5) | H16_A1331    |
| 14     | 1    | MenG2 demethylmenaquinone methyltransferase (EC 2.1.1.163) | H16_B0348    |
| 15     | 1    | MenG2 demethylmenaquinone methyltransferase (EC 2.1.1.163) | H16_B0348    |
| 16     | 1    | MenG2 demethylmenaquinone methyltransferase (EC 2.1.1.163) | H16_B0348    |
| 17     | 1    | PhaP1 Phasin (PHA-granule associated protein) | H16_A1381    |
| 18     | 1    | PhaP1 Phasin (PHA-granule associated protein) | H16_A1381    |
| 19     | 1    | PhaP1 Phasin (PHA-granule associated protein) | H16_A1381    |
| 20     | 1    | PhaP1 Phasin (PHA-granule associated protein) | H16_A1381    |

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The proteomic view on mutant R. eutropha PHB-4
Figure 3. Quantification of differentially expressed proteins in *R. eutropha* H16 and mutant *R. eutropha* PHB-4 as based on image fusion of 2D PAGE gels (Fig. 2). Spot quantities are given as % volume (representing the relative portion of an individual spot of the total protein present on the respective average fusion image). To facilitate comparison of spot quantities, panel A presents spots with relative quantities close to 1, while spots with higher quantities are shown in panel B. Quantification was done with Delta 2D software. Suffixes (a, b, and others) indicate isoforms of the same protein species present in the gels.
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above mentioned pyruvate dehydrogenase complex (PDHC), the 2-oxoglutarate dehydrogenase complex (OGDHC), the branched-chain dehydrogenase complex (BCDH), the glycine dehydrogenase complex (GDHC) and the acetoin dehydrogenase complex (ADHC) [74]. Regarding strain PHB-4, the flux of pyruvate from the KDPG pathway via PDHC to acetyl-CoA and finally to PHB is impaired, obviously leading to higher cellular amounts of acetyl-CoA and pyruvate that cannot be further converted. The observed overproduction of ThiJ might indicate a strengthening of the PDHC by providing greater amounts of cofactor TPP as an answer to an excess of pyruvate. It has been indirectly shown that PDHC plays the essential role in this context, as evidence has been provided that the pyruvate dehydrogenase, which harbours the cofactor TPP, can act as a bottleneck in the conversion of pyruvate. Thiamine auxotrophic mutants of Acinetobacter sp. as well as some other bacteria, which suffered from thiamine deficiency, excreted pyruvate to the medium in contrast to the wild types [75]. However, it is known that the PDHC is allosterically regulated with pyruvate acting as a positive effector and acetyl-CoA acting as a negative effector [74] which might counteract the metabolization of pyruvate by PDHC, if the acetyl-CoA concentration increases.

On the other hand, α- and β-subunits (AcoA, AcoB) of the E1 component of the acetoin dehydrogenase complex (ADHC) showed also higher synthesis in R. eutropha PHB-4. The ADHC shares as a member of the family of 2-oxoacid dehydrogenase complexes a related structure and also needs TPP as cofactor. It was shown that acetoin can be formed by a side reaction of the pyruvate decarboxylase (PdhA) of the PDHC (E1 component). In a first step, the conversion of hydroxyethyl-TPP to acetaldehyde with concomitant recovery of TPP occurs [76]. Furthermore, the PdhA is able to transfer the hydroxyethyl moiety of hydroxyethyl-TPP to acetaldehyde to finally form acetoin. This bypass might additionally counteract an accumulation of pyruvate which as an acid impairs the cell milieu, by providing an alternative path to acetyl-CoA, if PDHC was overloaded by abundant pyruvate and negatively affected by high concentrations of acetyl-CoA. Additionally, the interim accumulation of a certain amount of acetoin might be of particular advantage for the cells, as an overacidification of the intracellular environment and culture medium due to acidic product accumulation is prevented by conversion of pyruvate to uncharged acetoin [77].

Interestingly, our laboratory observed a similar effect on high cellular pyruvate concentrations in the context of a previous analysis of mutants of R. eutropha, which were lacking the dihydroliponamid dehydrogenase (PdhL), which constitutes the E3 component of the PDHC [78]. As the PDHC was in this case obviously not able to restore full activity by expression of homologues genes to pdhL encoded in Ralstonia’s genome, the cells likewise generated acetyl-CoA from pyruvate via ADHC and excreted excessive pyruvate.

The resulting acetyl-CoA subsequently enters the tricarboxylic acid cycle (TCC); the citrate synthase catalyzes the condensation reaction of acetyl-CoA and oxaloacetate to form citrate, which is further oxidized in the TCC. Oxaloacetate will be regenerated after the completion of one round of the TCC, or can be additionally provided by anaplerotic reactions [79]. The citrate lyase CitE4, which was found to be increasingly expressed in proteome analysis, catalyzes the reaction of citrate to acetate and oxaloacetate [63]. This reaction can obviously directly regenerate

Figure 4. Central metabolism of R. eutropha H16 and mutant PHB-4 with regard to the results of proteome analyses. The numbers in the scheme indicate the following involved enzymes: 1, glucokinase; 2, phosphogluconate dehydratase; 3, phospho-2-keto-3-desoxygluconate aldolase; 4, glyceraldehyde-3-phosphate dehydrogenase; 5, phosphoglycerate dehydrogenase; 6, phosphoglyceromutase; 7, enolase; 8, pyruvate kinase; 9, pyruvate dehydrogenase/decarboxylase (E1 of PDHC); 10, dihydrolipoamide acetyltransferase (E1 of PDHC); 11, dihydrolipoamide dehydrogenase (E3 of PDHC); 12, acetoin dehydrogenase enzyme system; 13, acetyl-CoA acetyltransferase; 14, acetoacetyl-CoA reductase; 15, PHB synthase; 16, 3-oxoacid-CoA transferase; 17, 3-hydroxybutyrate dehydrogenase; 18, citrate synthase; 19, aconitate; 20, isocitrate dehydrogenase; 21, 2-oxoacid dehydrogenase multienzyme complex; 22, succinyl-CoA synthetase; 23, succinate dehydrogenase; 24, fumarase; 25, malate dehydrogenase; 26, citrate lyase. doi:10.1371/journal.pone.0095907.g004
oxaloacetate and could therefore provide a direct anaplerotic link from citrate to oxaloacetate thereby circumventing the consecutive reaction steps of the TCC. Larger quantities of oxaloacetate are probably needed for the conversion of the enhanced amounts of acetyl-CoA which can not be directed towards PHB synthesis in mutant PHB-4. Indeed, experiments of Ruhr [80] showed a 2.2-fold higher intracellular concentration of acetyl-CoA in autotrophic cells of mutant PHB-4 when ammonium was absent. The corresponding expected lower intracellular concentration of free CoA in turn negatively influences the catalytic rate of the pyruvate dehydrogenase and supports excretion of pyruvate [73].

The upregulated kinase ThiJ may likewise enhance the activity of the OGDHC (or alternatively α-ketoglutarate dehydrogenase complex), which in a later step decarboxylates α-ketoglutarate to succinyl-CoA with concomitant reduction of NAD⁺, since TPP acts also as cofactor in this multi-enzyme complex. Succinyl-CoA is subsequently converted so succinate by means of the succinyl-CoA synthetase under generation of GTP. Interestingly, a succinyl CoA-transferase [H16_A1331], which catalyzes the transfer of coenzyme A from succinyl-CoA to acetoclastic or vice versa [66], showed much stronger expression in strain PHB-4. This indicates that this transferase may play a role in transferring CoA from acetoclastic-CoA to succinate in order to reduce the acetocetyl-CoA pool, because the latter will accumulate in mutant PHB-4 as a direct precursor of PHB due to the lack of an active PHA synthase in this strain.

Furthermore, five isoforms of two different acyl-CoA transferases (H16_A2386, H16_B2114) were observed among the upregulated proteins. Generally, CoA-transferases catalyze reversible transfer reactions of coenzyme A from CoA-thioesters to free acids [57] and can exhibit relative broad substrate specificities [81]. The precise donor and acceptor molecules of these two acyl-CoA transferases of R. eutropha are unknown, yet. However, the significant increased synthesis of acyl-CoA transferases in mutant PHB-4 indicates a need for transfer reactions between organic acid intermediates as a response of impaired PHB accumulation.

Two demethylmaenaquinone methyltransferases, MenG2 and MenG3, which catalyze the carbon methylation reaction during biosynthesis of ubiquinone (coenzyme Q) and menaquinone (vitamin K2), were significantly stronger synthesized in strain PHB-4. Both isoprenoid quinones are essential components of the oxidative stress caused by the loss of PHB synthesis in mutant PHB-4. Indeed, experiments of Ruhr [80] showed a 2.2-fold higher intracellular concentration of acetyl-CoA in turn negatively influences the catalytic rate of the pyruvate dehydrogenase and supports excretion of pyruvate [73].

Mutant PHB-4 tries to prevent acidification by (a) excretion of pyruvate and by (b) stronger synthesis of the observed protein species: Increased expression of (i) ThiJ supports biosynthesis of cofactor TPP and thereby reinforces the 2-oxoacid dehydrogenase complexes PDHC, ADHC and OGDHC in order to convert pyruvate at a higher rate. Additionally, the (ii) 3-isopropylmalate dehydrogenase LeuB3 may direct pyruvate into synthesis of different amino acids. Larger amounts of acetyl-CoA that cannot be channeled to PHB synthesis in mutant PHB-4 are countered by increased synthesis of (iii) different acylCoA-transferases which allow transfer reactions between organic acid intermediates. A large fraction of acetyl-CoA enters the TCC, and (iv) the increasingly expressed citrate lyase CitE4 can regenerate oxaloacetate from citrate for the conversion with acetyl-CoA in a kind of direct anaplerotic link. Substantial amounts of reduction equivalents that emerge inTCC are countered by (v) enhancing capacity of the respiratory chain through synthesis of more ubiquinones as indicated by the observed stronger synthesis of MenG2 and MenG3. Finally, the (vi) stronger expressed glutathione transferase SspA could reduce oxidative stress caused by the loss of PHB synthesis in mutant PHB-4.

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Author Contributions

Conceived and designed the experiments: MR. Performed the experiments: MR. Analyzed the data: MR. Contributed reagents/materials/analysis tools: BV MH. Wrote the paper: MR. Performed the MALDI-TOF analysis: BV MH. Supervision: AS. Revised the manuscript: AS.

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