Studies on the aerobic utilization of synthesis gas (syngas) by wild type and recombinant strains of Ralstonia eutropha H16

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Summary

The biotechnical platform strain Ralstonia eutropha H16 was genetically engineered to express a cox subcluster of the carboxydotrophic Oligotropha carboxidovorans OM5, including (i) the structural genes coxM, -S and -L, coding for an aerobic carbon monoxide dehydrogenase (CODH) and (ii) the genes coxD, -E, -F and -G, essential for the maturation of CODH. The coxOc genes expressed under control of the CO2-inducible promoter P1 enabled R. eutropha to oxidize CO to CO2 for the use as carbon source, as demonstrated by 13CO experiments, but the recombinant strains remained dependent on H2 as external energy supply. Therefore, a synthetic metabolism, which could be described as ‘carboxyhydrogenotrophic’, was established in R. eutropha. With this extension of the bacterium’s substrate range, growth in CO2, H2 and CO2-containing artificial synthesis gas atmosphere was enhanced, and poly(3-hydroxybutyrate) synthesis was increased by more than 20%.

Introduction

The β-proteobacterium Ralstonia eutropha H16, currently named as Cupriavidus necator H16, represents a model organism for autotrophic lifestyle and has been studied extensively for its efficient utilization of carbon dioxide and hydrogen as carbon and energy sources (Friedrich and Schwartz, 1993). By means of enzymes of the Calvin-Benson-Bassham (CBB) cycle, R. eutropha H16 fixes CO2 from the atmosphere as the sole source of carbon as well as from CO2, generated from its own intracellular oxidation of organic carbon compounds like formate (Kärst and Friedrich, 1984; Shimizu et al., 2015). When growing lithotrophically, R. eutropha H16 oxidizes molecular hydrogen by a soluble and a membrane-bound hydrogenase, which are tolerant to oxygen, as well as to carbon monoxide (Buhrike et al., 2005; Burgdorf et al., 2005; Bürstell et al., 2016).

Several approaches have been made to exploit R. eutropha’s efficient chemolithoautotrophic lifestyle to potentially capture CO2 from waste- or industrial exhaust gases for the conversion to various products of increased value. These so-called second-generation bioproducts include polymers, fuels and fine chemicals (Cook and Schiegel, 1978; Tanaka and Ishizaki, 1994; Müller et al., 2013; Lu and Yu, 2017). An alternative form of a gaseous feedstock that can be produced from fossil as well as from renewable resources, is the platform chemical synthesis gas (syngas), which has gained considerable attention for the use as cheap, energy-rich and abundant feedstock for microbial processes in recent years (Drzyzga et al., 2015). Besides hydrogen and some CO2, a major component of syngas is carbon monoxide, which can be utilized as carbon and energy source by various bacteria. These include the so-called aerobic carboxydotrophs, such as Oligotropha carboxidovorans and Alcaligenes carboxydotius, which exhibit notable physiological similarities to R. eutropha (Cypionka and Meyer, 1982; King, 2003). Carboxydotrophic bacteria oxidize CO with H2O to CO2 and 2 H+ + 2 e− by a carbon monoxide dehydrogenase (CODH), thereby obtaining energy and carbon, that is usually fixed in the CBB cycle. The CODH-associated proteins of the model organism for carboxydotrophy, O. carboxidovorans OM5, are encoded in a single 14.5-kbp cox gene cluster, which is located on the organism’s megaplasmid pHCG3 (Fig. S1; Fuhrmann et al., 2003). This cluster includes the structural genes for CODH, coxM, coxS and coxL, as well as genes that code for proteins mediating the post-translational maturation of CODH (coxD, coxE, coxF, coxG; Schübel et al., 1995). The remaining coxOc...
genes, which encode transmembrane complexes (\textit{coxB}, \textit{coxK}, \textit{coxI}) and a putative signal transduction system (\textit{coxC}, \textit{coxH}), have yet to be further characterized.

Although a utilization of carbon monoxide as a source of carbon or energy has not been shown, \textit{R. eutropha} H16 has several characteristics, which suggest that a possible exploitation of the energy-rich substrate CO by this bacterium is not far off. Similarly to its two hydrogenases, examined cytochromes of \textit{R. eutropha} H16 showed low affinity towards CO (Bernard et al., 1974). This has led to the assumption that the respiratory system of \textit{R. eutropha} H16 is mostly CO-insensitive, which was supported by the study of Cypionka and Meyer (1982), who observed a substantial inhibition of growth of \textit{R. eutropha} only at CO concentrations that exceeded those of conventional syngas by far. Furthermore, the two chromosomes of \textit{R. eutropha} H16 harbour putative genes with significant similarities to \textit{cox} genes of \textit{O. carboxidovorans} (Cramm, 2009). Recently, \textit{R. eutropha} was genetically engineered to display a cell surface-anchoring protein consisting of CODH subunits of different anaerobic bacteria that converted CO to CO$_2$ (Hyeon et al., 2015). However, an intracellular utilization of CO by \textit{R. eutropha}, resulting in growth, has not been shown to this date. In this study, cultivations of \textit{R. eutropha} H16 with a standardized artificial syngas mixture or different, defined compositions of CO, CO$_2$ and H$_2$ were carried out and the effect of heterologously expressed \textit{cox} genes of \textit{O. carboxidovorans} OM5 on growth in CO-containing gas mixtures by \textit{R. eutropha} was examined.

Results and discussion

\textit{Cultivation of strains of \textit{R. eutropha} in the presence of carbon monoxide}

To assess and enhance growth of \textit{R. eutropha} H16 in CO-containing atmospheres, cells of the wild type and of recombinant strains were initially cultivated aerobically with 30\% (by volume) of an artificial syngas mixture. This gas mixture consisted of (by volume) 40\% CO, 40\% H$_2$, 10\% CO$_2$ and 10\% N$_2$, and resembled syngas compositions, which were previously used in academic studies and were actually obtained from gasification of biomass (Bridgewater, 1995; Heinrich et al., 2016; Revelles et al., 2016). To improve the utilization of syngas, of which CO is a major component, seven \textit{cox} genes of \textit{O. carboxidovorans} OM5 were heterologously expressed in \textit{R. eutropha} H16. For this, \textit{coxM}, \textit{-S}, \textit{-L}, \textit{-D}, \textit{-E}, \textit{-F} and \textit{-G} (locus tags: OCA5\_RS17205-260), which are the conserved \textit{cox} genes among carboxydrotrophs and are regarded as essential for carbon monoxide utilization (Santiago et al., 1999; King and Weber, 2007), were cloned to yield the construct pBBR1MCS-3::\textit{coxMSLDEFG}$_{Oc}$. Furthermore, the CO$_2$-inducible promoter P$_L$ was cloned upstream of the \textit{coxOc} genes to exploit the gas exposure for enhanced expression. This promoter mediates gene transcription of the two homologous \textit{cbb} operons of \textit{R. eutropha} and binds the transcription activator CbbR (Jeffke et al., 1999). Syngas cultures of \textit{R. eutropha} showed exponential growth after an initial lag phase of three to four days, which showed that all glucanase of the precultures had been successfully washed out from the medium prior to inoculation (Fig. 1). From day five onwards, the gas phase was renewed with every sample withdrawal to prevent a lack of carbon, or energy supply for these cultures. Recombinant \textit{R. eutropha} strains, which harboured the \textit{coxMSLDEFG}$_{Oc}$ cluster, showed substantially enhanced growth in particular during the first 10 days of cultivation (Table 1) when compared to the control strains, which carried the plasmids pBBR1MCS-3 or pBBR1MCS-3::\textit{cox}. The strain harbouring pBBR1MCS-3-P$_L$::\textit{coxMSLDEFG}$_{Oc}$ grew to the highest optical density (OD$_{600nm}$: 5.48). The increased optical densities were not only a result of cell growth, as depicted by the measured cell dry weights (CDW), but also of the accumulated amounts of the carbon storage compound poly(3-hydroxybutyrate) (poly[3HB]), which were increased by more than 20\% concomitant with heterologous expression of the \textit{coxOc} genes (Table 1). The crystalline short chain length polymer poly[3HB] is known to considerably affect the
transmission of light through the cell material (Schlegel et al., 1970). Due to the technical limitation of the applied proof-of-concept set-up, the exact amounts of utilized CO and CO₂ from applied syngas were not determined. However, by means of unpaired t-tests, the impact of the heterologously expressed coxMSLDEFGOc genes on growth and poly(3HB) synthesis of the respective R. eutropha strains (Table 1) was determined to be significant for all analysed parameters (P < 0.05), which suggested an increased utilization of carbon, CO and/or CO₂, which was present in the artificial syngas mixture.

To verify this assumed utilization of CO, respective strains were cultivated in a similar set-up, in which cells were cultivated with syngas for 5 days, harvested and washed with medium and then transferred to flasks containing fresh media with an aerobic atmosphere containing (i) 10% CO with 10% H₂, (ii) 10% CO, or (iii) 10% H₂ (by volume). To gain sufficient amounts of cell matter for poly(3HB) analysis without extending the cultivation time of the previous growth experiment (Fig. 1), the cells were initially inoculated to a higher OD₆₀₀nm. The difference in growth during the first 5 days of cultivation between R. eutropha pBBR1MCS-3::coxMSLDEFGOc and its corresponding control strain (empty vector) appeared to be less substantial than during the first days of the previous syngas cultivation (Fig. 1). Taking the higher concentrations of the inocula into account, it could be assumed that the positive effect of the vector pBBR1MCS-3::coxMSLDEFGOc on growth with syngas was less noticeable with increasing OD₆₀₀nm (Fig. 1, d 10–15; Fig. 2, d 0–5). At these higher cell densities, the comparatively lower amount of CO substrate available for each individual cell, combined with the presumably low concentration of recombinant intracellular CODH_Oc, resulting from basal gene expression through P_lac, might have led to a relatively low rate of conversion of CO to CO₂ for growth.

Upon the exchange of atmospheres, none of the four tested strains of R. eutropha was able to grow, when solely CO or H₂ were applied (Fig. S2). In contrast, cells showed a slight, but significant increase in optical density, when 10% H₂ was added to 10% of CO (Fig. 2). Again, R. eutropha pBBR1MCS-3-PcoxMSLDEFGOc appeared to be the best-performing strain and gained nearly 0.5 units of OD₆₀₀nm within 20 days of cultivation with CO/H₂.

These cultivations suggested that the strains of R. eutropha expressing the seven cox genes of O. carboxidovorans OM5 were able to utilize carbon monoxide as carbon source but were unable to channel the resulting electrons into the respiratory chain for the generation of energy, as provision of H₂ was essential for an increase of the OD. Surprisingly, also the control strains harbouring the respective vectors without coxOc genes showed a slight increase in OD₆₀₀nm, however, to a much lower degree as compared to the recombinant strains. In these two-phase cultivation experiments, the cells accumulated considerably less poly(3HB) when compared to the previous cultivations, in which syngas was applied over the entire course of the cultivation (Table 1). As syngas contained 10% of CO₂, which in contrast to CO can be immediately assimilated through the CBB cycle, the amount of available carbon, crucial for poly(3HB) accumulation in R. eutropha, was likely higher when the
strains were cultivated in syngas atmosphere (Anderson and Dawes, 1990). Furthermore, strains of *R. eutropha*, which harboured the *coxOc* genes, accumulated only slightly more poly(3HB) (*coxMSLDEFGOc*: 18.1% ± 1.1%; *P<sub>L</sub>*: *coxMSLDEFGOc*: 18.9% ± 0.9% [wt/wt of CDW]) than the corresponding control strains (empty vector: 17.0% ± 0.3%; pBBR1MCS-3-*P<sub>L</sub>*: 16.7% ± 0.5% [wt/wt of CDW]). This implied that the increased optical density of these recombinant strains was not only due to light scattering of the accumulated polymer granules, but also to cell division.

Remarkably, no growth was detected when cells were directly exposed to the aerobic CO/H<sub>2</sub>-containing atmosphere after inoculating the cells from the heterotrophically grown precultures. An explanation for this might be the display of cytochrome patterns by *R. eutropha*, which evidently vary according to different growth phases and conditions (Kőmen et al., 1991). No experiments on the cytochrome CO sensitivity of heterotrophically grown cells of *R. eutropha* H16 have been carried out; however, Probst and Schlegel (1976) reported a moderate affinity of cytochromes towards CO in CO<sub>2</sub>/H<sub>2</sub>-cultivated cells of this bacterium. Therefore, one could speculate that cells of *R. eutropha* that were grown in syngas prior to the switch to CO/H<sub>2</sub> atmosphere built a cytochrome pattern, which was more adapted to CO exposure (Fig. 2).

In both cultivation experiments, the applied *P<sub>L</sub>* promoter had a positive impact on growth of the recombinant *R. eutropha* strain, which harboured the *coxOc* genes. This was most likely due to an increased level of expression of the episomal heterologous genes that was caused by the induced *P<sub>L</sub>* promoter. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) protein patterns of separated soluble supernatants of disrupted syngas-grown cells did not show notable differences in occurrence or abundance of proteins between the recombinant *coxOc* strains and the *R. eutropha* H16 wild type, harbouring the empty vector (Fig. S3). However, the protein pattern of the membrane-containing pellet of *R. eutropha* pBBR1MCS-3-*P<sub>L</sub>*::*coxMSLDEFGOc* (Fig. 3) displayed a distinct spot that matched the size of the large CODH subunit Cox<sub>L</sub> (88.7 kDa; Dobbek et al., 1999). Applying matrix-assisted laser desorption/ionization-time-of-flight-tandem-mass spectrometry (MALDI-TOF-MS/MS) and subsequent data analysis with the Mascot search engine, this protein spot was clearly identified as Cox<sub>L</sub> (Protein score, 105.5; Confidence interval [C.I.%], 100%). Other Cox subunits or accessory proteins could not be detected; this may be due to (i) an overlay of proteins, (ii) weakspecific binding of the Coomassie dye or (iii) partial 3′-terminal degradation of mRNA of a respective gene, downstream of *coxL*</sub><sub>Oc</sub>. Moreover, the expression of *coxOc* genes under control of the pBBR1MCS-3 standard *P<sub>lac</sub>* promoter, which cannot be induced but exhibits basal activity in *R. eutropha* strains, presumably occurred at a low level. This led to protein concentrations that cannot be detected in SDS PAGE protein patterns.

The detection of at least the Cox<sub>L</sub>-Oc subunit in disrupted cells of *R. eutropha* pBBR1MCS-3-*P<sub>L</sub>*::*coxMSLDEFGOc* together with the increased growth in CO-containing atmospheres of this strain indicated that the applied *P<sub>L</sub>* is a suitable promoter for heterologous gene expression in syngas cultivations with *R. eutropha*. The CO<sub>2</sub>, which was responsible for induction of the *P<sub>L</sub>* promoter, was solved in the culture broth and additionally emerged from the assumed intracellular conversion of CO to CO<sub>2</sub>. In contrast, the presence of the *P<sub>L</sub>* promoter on the control plasmid pBBR1MCS-3-*P<sub>L</sub>* leads to decreased growth (Figs 1 and 2). As episomal copies of *P<sub>L</sub>* could capture the regulating CbbR proteins, which are required for transcription of the *cbb* genes of
**Aerobic syngas utilization by Ralstonia eutropha**

**Ralstonia eutropha**, CO₂ fixation of the respective strain might have been hampered (Bowien and Kusian, 2002).

As an additional observation, the positive impact of the coxOC genes on growth of the recombinant *R. eutropha* strains in CO-containing atmospheres was more substantial in syngas atmosphere (Fig. 1) than in the two-phase cultivation, where CO was the sole carbon source (Fig. 2). Possibly, the presence of CO₂, which is a natural substrate of *R. eutropha*, leads to an increased fitness of the recombinant strains as compared to cultivations, where cells solely relied on carbon monoxide. As a mere hypothesis, the recombinant CODHOC could not only provide limited amounts of CO₂ as carbon source for *R. eutropha*, but as a side-effect could also dispose the medium of accumulating CO, which would be partially inhibiting growth at otherwise increasingly higher concentrations (Cypionka and Meyer, 1982). Despite their inability to gain sufficient amounts of energy from carbon monoxide, this possible detoxification effect could explain the enhanced productivity of the recombinant *R. eutropha* coxOC strains in the conducted syngas cultivations, as compared to the wild type.

**In vitro analysis of carbon monoxide oxidation by strains of R. eutropha H16**

To demonstrate the ability of the recombinant *R. eutropha* strains to oxidize CO to CO₂ in *vitro*, an altered protocol of the photometric assay of Meyer and Schlegel (1978), in which the oxidation of CO is coupled to the reduction of methylene blue, was applied. For this, duplicate flasks with cells of the different generated strains of *R. eutropha* were cultivated in 30% of syngas atmosphere (Fig. 1) than in the two-phase cultivation, where CO was the sole carbon source (Fig. 2). Possibly, the presence of CO₂, which is a natural substrate of *R. eutropha*, leads to an increased fitness of the recombinant strains as compared to cultivations, where cells solely relied on carbon monoxide. As a mere hypothesis, the recombinant CODHOC could not only provide limited amounts of CO₂ as carbon source for *R. eutropha*, but as a side-effect could also dispose the medium of accumulating CO, which would be partially inhibiting growth at otherwise increasingly higher concentrations (Cypionka and Meyer, 1982). Despite their inability to gain sufficient amounts of energy from carbon monoxide, this possible detoxification effect could explain the enhanced productivity of the recombinant *R. eutropha* coxOC strains in the conducted syngas cultivations, as compared to the wild type.

**Cultivation of strains of R. eutropha H16 with ¹³CO**

To demonstrate that the *R. eutropha* coxOC strains were not only able to oxidize carbon monoxide but also to assimilate the CO-derived carbon, the strains were cultivated with ¹³CO-labelled carbon monoxide as the sole C source. For this purpose, a shortened two-stage cultivation was carried out, which comprised 5 days of growth with the artificial syngas mixture followed by 10 days of exposure to (by volume) 10% ¹³CO + 10% H₂ (Fig. S4). Along with the cell numbers of withdrawn samples, conversion of ¹³CO into poly(3HB) was determined by gas chromatography–mass spectrometry (GC-MS), as poly (3HB) derives from the central metabolite acetyl coenzyme A (acetyl-CoA). Therefore, ¹³C enrichment in the characteristic m/z 103 fragment of methanolyzed poly (3HB) was determined (Table 2). The recombinant coxOC strains of *R. eutropha* H16 again showed only a slight increase in optical density, but incorporated substantial amounts of ¹³C-labelled carbon, which derived from the applied ¹³CO, into the backbone of the accumulated poly(3HB) after 10 days of cultivation with ¹³CO (Table 2). Taking the natural abundance of carbon, oxygen and hydrogen isotopes into account, most of these labelled monomers appeared to contain three ¹³C atoms (m/z: 106), whereas only a small amount of monomers containing one ¹³C atom (m/z: 104) and no monomers.
Table 2. Changes in optical density, cell number and poly(3HB) accumulation as well as incorporation of $^{13}$C into poly(3HB) of generated strains of *Ralstonia eutropha* H16 after ten days of cultivation with 80% air, 10% $^{13}$CO and 10% H$_2$ (by volume). Cells were initially grown in 100 ml mineral salts medium (Schlegel et al., 1961) at 30 °C and 130 r.p.m. under oxic conditions in 1 L Duran flasks with an atmosphere of 30% of an artificial synthesis gas mixture (by volume 40% CO, 40% H$_2$, 10% CO$_2$, 10% N$_2$). After five days of cultivation, 50 ml of culture broth was harvested, and the remaining 50 ml was washed and transferred to fresh media. Cultures were then set to an atmosphere of 80% air, 10% $^{13}$CO and 10% H$_2$ (by volume), which was renewed after five further days of cultivation, before the remaining cultures were harvested. Data were obtained from duplicate flasks. M0 to M3 display the m + 0 to m + 3 enrichments of the 3HB-methyl ester’s m/z 103 fragment with $^{13}$C, detected by GC-MS as described by Tan et al. (2016). The fractions of $^{13}$C-enriched 3HB constituents were determined after correcting for natural isotope abundances.

| R. eutropha pBBR1MCS-3: | $\Delta$ OD$_{600\text{nm}}$ | $\Delta$ Cell number (10$^8$ ml$^{-1}$) | $\Delta$ poly(3HB) (wt/wt of CDW) | Incorporation of $^{13}$C (M0/M1/M2/M3) |
|-----------------------------|-----------------------------|----------------------------------|---------------------------------|----------------------------------|
| (Only vector)               | 0.08                        | 0.21                             | 0.2%                            | 99.8/0.2/2/--/--                 |
| $P_L$                       | ±0.02                       | ±0.11                            | ±0.1%                           | ±0.2/0.2/2/--/--                 |
| $coxMSLDEFG_Oc$             | ±0.06                       | ±0.05                            | ±0.1%                           | 100/1/0/0/0                      |
| $P_L$: $coxMSLDEFG_Oc$      | ±0.00                       | ±0.05                            | ±0.1%                           | ±0.0/0/0/0/0                     |
| $P_L$: $coxMSLDEFG_Oc$      | ±0.00                       | ±0.13                            | ±0.4%                           | ±0.6/0.2/2/--/0.4                |
| $P_L$: $coxMSLDEFG_Oc$      | ±0.04                       | ±0.19                            | ±0.5%                           | ±0.8/1.0/2/0.2                   |

containing two $^{13}$C (m/z 105) atoms were detected (Fig. S5). As cultures were supplied with exclusively $^{12}$C containing syngas (d 0–5) or $^{13}$CO (d 5–15), the occurrence of polymer constituents containing both $^{12}$C and $^{13}$C atoms could have resulted from $^{12}$C which was mobilized from the central metabolism, biomass or poly(3HB) during cultivation with $^{13}$CO (Table 2). This may be due to the simultaneous synthesis and degradation of poly(3-hydroxyalkanoate) by *R. eutropha* H16, first reported by Doi et al. (1990). The 3HB constituents originating from non-labelled carbon compounds of syngas were thereby steadily replaced by constituents derived from $^{13}$CO. Furthermore, the cell number of the strains, harbouring $cox_{Oc}$ genes slightly increased over the course of the exposure to $^{13}$CO and H$_2$ (Table 2), which suggested that carbon from CO was utilized for cell growth. A minor increase in cell number was also recorded for the *R. eutropha* control strains harbouring the empty pBBR1MCS-3(-$P_L$), which, however, did not meet statistical significance ($P > 0.05$). The pH of the cultures after 15 days of cultivation with $^{13}$CO/H$_2$ was in the range of 6.6 (± 0.2), whereas cultures displayed an approximate pH of 6.7 (± 0.1) after the five-day syngas cultivation phase, which implied that this parameter did not affect growth with CO and H$_2$. These results, combined with the foregoing growth experiments and the photometric enzyme assay, provided unequivocal evidence that the recombinant *R. eutropha* strains generated in this study were able to oxidize and utilize CO as the sole carbon source by CODH$_{Oc}$-mediated conversion to CO$_2$.

To this point, the cause for the inability to sufficiently exploit the reducing power of CO for cell growth remains unclear but cannot be experimentally solved within this study. A critical point when discussing the transfer of electrons from the CODH$_{Oc}$ to the respiratory chain is the identification of the responsible electron acceptor. Although cytochrome $b_{661}$ was widely considered to accept the reducing equivalents from the CODH of *O. carboxidovorans* (Cypionka and Meyer, 1983), recent experiments suggested that the common redox carrier ubiquinone (coenzyme Q10) initially interacts with the CODH flavin site (Wilcoxen et al., 2011). Furthermore, sequence analysis revealed that *R. eutropha* H16 possesses a cytochrome $b$ large subunit (HoxZ) with a high identity to cytochrome $b_{661}$ of *O. carboxidovorans* OM5 (BLASTP expect value, 4e$^{-63}$; identities, 147/242 [60%]; positives, 185/242 [76%]; gaps, 3/242 [1%]; Altschul et al., 1997). Therefore, *R. eutropha* could potentially transfer reducing equivalents from CO to its respiratory chain either way. Possibly, an imperfect insertion of the recombinant CODH into the cytoplasmic membrane sterically hindered the transmission of reducing equivalents to their acceptor in the *R. eutropha* $cox_{Oc}$ strains. In this case, the fate of the electrons of the reduced CODH would be unclear at this point. A simpler explanation would be that the amount of CO, which is oxidized by the recombinant CODH$_{Oc}$, is insufficient to feed the energy demand of the cell, for instance, CO$_2$ fixation through the CBB cycle. This was implied by the considerably lower CO oxidation rates of the recombinant *R. eutropha* strains as compared to the reference *O. carboxidovorans* OM5. Several bacteria, e.g. of the genus *Mesorhizobium*, can oxidize CO but are unable to utilize the resulting CO$_2$ for cell growth, due to the absence of CO$_2$-fixing mechanisms and are thus referred to as ‘carboxydovores’ (King, 2003). In contrast to these lithoheterotrophic ‘carboxydovores’, the recombinant strains of *R. eutropha* cultivated in this study metabolized CO
as source of carbon, but both were dependent on molecular hydrogen for cell growth. Consequently, the CO-related lifestyle of the engineered strains of R. eutropha could be referred to as ‘carboxyhydrogenotrophic’.

No in vitro CO oxidation was detected for the R. eutropha control strains harbouring the empty pBBR1MCS-3 or pBBR1MCS-3-P L, which consequently were therefore either unable to oxidize CO under the applied conditions, or their reactions were below the detection level respectively. Previous studies by King and Weber (2007), as well as Cramm (2009) reported the presence of multiple coxL homologues in the genome of R. eutropha H16. Of these, one putative coxL gene (locus tag: H16_RS02155) is located in a coxSLMDEGI cluster, which was shown to be constitutively transcribed, at least under heterotrophic growth conditions, in foreg- ing, studies by Peplinski et al. (2010). This cluster was annotated to putatively encode a xanthine oxidase, an enzyme that has diverse substrate specificities and is phylogenetically close to CODHs of, e.g. O. carboxidovorans (King, 2003; Hille, 2005). Still, a possible activity of this putatively formed enzyme towards carbon monoxide remains to be investigated in further studies.

Due to its metabolic versatility, R. eutropha H16 has been exploited to synthesize biobased products from various heterotrophic and autotrophic carbon sources (Volodina et al., 2016). Yet CO has been widely ignored as substrate for this bacterium, despite the importance of utilizing all carbon compounds of waste gases for a desirable circular economy. In this study, the utilization of CO as part of the economically and ecologically sustainable feedstock syngas by R. eutropha H16 through heterologous expression of the coxSLMDEGI genes of O. carboxidovorans OM5 has been demonstrated both in vivo and in vitro. A conceivable benefit of this COD-HOC- mediated oxidation of CO over the recently developed cell surface-attached CODH (Hyeon et al., 2015) could be the intracellularely nascent CO2 that is readily available to be incorporated into the CBB cycle by the ribulose 1,5 bisphosphate carboxylase. In contrast, extracellular CO2 enters the cell as HCO3− and thus initially must be converted by the R. eutropha carbonic anhydrase Cag (Gai et al., 2014). By oxidation of toxic CO to CO2 and the resulting supply of additional accessible carbon, potential syngas processes involving R. eutropha H16, could exhibit enhanced productivity and efficiency, as shown for the commercially applicable biopolymer poly(3HB) in this study.

**Experimental procedures**

**Microorganisms, plasmids and oligonucleotides**

Bacterial strains, plasmids and oligonucleotides, which were used in this study, are listed in Table S1.

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**Construction of vectors and generation of recombinant strains of E. coli and R. eutropha**

Nucleic acids were processed according to Sambrook et al. (1989). DNA fragments were amplified from...
genomic DNA of *O. carboxidovorans* OM5 and *R. eutropha* H16 by applying the Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) with oligonucleotides, listed in Table S1. FastDigest restriction enzymes (Thermo Scientific, Waltham, MA, USA) were used to digest DNA fragments, before ligation into a likewise digested target plasmid applying T4 DNA ligase (Thermo Scientific, Waltham, MA, USA). To propagate, isolate and validate generated hybrid plasmids, chemically competent cells of *E. coli* TOP10 were transformed, applying the method of Hanahan (1983). Re-isolated plasmids were then transferred into *R. eutropha* H16 by electroporation (Aneja et al., 2009). The vector pBBR1MCS-3::coxMSLDEFGoC was generated by digesting the amplified *coxMSLDEFGoC* DNA fragment with SpeI and SacI, and subsequent ligation into a likewise digested pBBR1MCS-3 vector. SpeI-digested P$_r$ fragments were then inserted into the corresponding site to yield the vector pBBR1MCS-3-P$_r$::coxMSLDEFGoC.

**Preparation of cell extract and analysis of protein patterns**

Harvested cells were washed and resuspended in 50-mM KH$_2$PO$_4$-KOH buffer (pH 7.0) with added protease inhibitor (Complete ULTRA Tablets, Roche, Basel, CH), and cell extracts of *R. eutropha* were obtained by sonication and subsequent centrifugation (10 min, 13 000 g, 4 °C). Protein concentrations were determined employing the method of Bradford (1976), and 40 μg of protein were heated in denaturing buffer for 10 min at 95 °C. Upon separation by SDS PAGE (Laemmli, 1970), proteins were stained with Coomassie brilliant blue R-250. Proteins were identified by MALDI-TOF-MS/MS. For this, respective protein spots were excised from SDS gels and transferred to 1.5 ml reaction tubes containing 50 μl 10% (vol/vol) acetic acid. Protein samples were subjected to MALDI-TOF-MS/MS analysis as described by Wolf et al. (2008). Applying a 5800 Proteomics Analyzer (AB Sciex, Framingham, MA, USA), the spectra were recorded in a reflector mode in a mass range from 900 to 3700 Da with a focus mass of 200 Da. Acquired MS data were compared to the proteome database of *O. carboxidovorans* OM5 and *R. eutropha* H16 using the Mascot engine (version 2.1.0.4).

**Determination of CODH activity in cell extracts**

CODH activity in cell lysates was determined with a method modified according to Meyer and Schlegel (1978). In the present protocol, the oxidation of glucose by a glucose oxidase/catalase mix to dispose residual oxygen was omitted, as it affected the reduction of methylene blue from CO in the applied experimental set-up. The reaction was started by adding 20 μl of soluble cell extract (2–8 mg ml$^{-1}$ of protein) or 1–3 mg of resuspended wet cell pellet to 1.8 ml of the CO–saturated 50 mM methylene blue solution.

**Analysis of poly(3HB) content of cells**

Upon cell harvest, cells were lyophilized, and 5–10 mg of each sample was subjected to acidic methanolation as described by Brandl et al. (1988). Synthesized 3HB-methyl esters were then quantified by gas chromatography (Timm et al., 1990), using a Agilent 6850 gas chromatograph (GC), which was equipped with a BP21 polyethylene glycol capillary column (50 m by 0.22 mm; 250 nm film thickness) and a flame ionization detector (Agilent Technologies, Waldbronn, D). To determine the content of $^{13}$C, obtained 3HB-methyl esters were analysed by GC-MS according to Tan et al. (2016). Applying an Agilent 6890 GC, which was connected to an Agilent HP 5973 mass spectrometer (MS), the samples were separated on a BPX35 polyethylene glycol capillary column (60 m by 0.22 mm; 250 nm film thickness; SGE Deutschland GmbH, Darmstadt, D). For this, the temperature programme described by Andreeßen et al. (2010) was applied.

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**Conflict of interest**

The authors have no conflict of interest to declare.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Table S1.** Microorganisms, target vectors and oligonucleotides, which were applied in this study.

**Fig. S1.** Cluster of cox genes in *Oligotropha carboxidovorans* OM5.

**Fig. S2.** Cultivation of strains of *R. eutropha* H16, harbouring pBBR1MCS-3 (empty vector), pBBR1MCS-3::coxMSLDEFGOc or pBBR1MCS-3-PcoxMSLDEFGOc, in 1L Duran flasks filled with 50 ml mineral salts medium (Schübel *et al.*, 1961) at 30 °C and 130 r.p.m.

**Fig. S3.** Protein patterns of the soluble supernatant of lysed cells of *R. eutropha*, harbouring pBBR1MCS-3::coxMSLDEFGoc (A), pBBR1MCS-3-PcoxMSLDEFGoc (B); or pBBR1MCS-3 (empty vector; C).

**Fig. S4.** Cultivation of strains of *R. eutropha* H16, harbouring pBBR1MCS-3 (empty vector), pBBR1MCS-3-PcoxMSLDEFGoc or pBBR1MCS-3-PcoxMSLDEFGoc in 1L Duran flasks at 30 °C and an agitation of 130 r.p.m.

**Fig. S5.** Mass spectra of 3-hydroxybutyrate methyl esters, extracted from cells of *Ralstonia eutropha* pBBR1MCS-3-PcoxMSLDEFGoc.