RESEARCH LETTER

Impact of the small RNA RyhB on growth, physiology and heterologous protein expression in *Escherichia coli*

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Received 16 May 2007; revised 6 July 2007; accepted 11 July 2007.
First published online September 2007.

DOI:10.1111/j.1574-6968.2007.00880.x

Editor: Diethard Mattanovich

Keywords
RyhB; small RNA; iron; protein expression; *Escherichia coli*.

Abstract

The small noncoding RNA RyhB is a regulator of iron homeostasis in *Escherichia coli*. During iron limitation, it downregulates the expression of a number of iron-containing proteins, including enzymes of the tricarboxylic acid cycle and the respiratory chain. Because this infers a potential for RyhB to limit energy metabolism and biosynthetic capacity, the effect of knocking out *ryhB* on the physiology and heterologous protein productivity of *E. coli* has been analyzed. During iron limitation, induced either through insufficient extracellular supply or through overexpression of an iron-containing protein, *ryhB* mutants showed unaltered growth and substrate consumption. They did, however, exhibit significantly lowered acetate production rates. Plasmid-based expression of green fluorescent protein and the heterologous *Vitreoscilla* hemoglobin VHb was negatively affected by the *ryhB* knock-out.

Introduction

Escherichia coli has become established not only as one of the most important hosts for the commercial production of biopharmaceutical proteins (Walsh, 2006) but also as an indispensable tool to express recombinant proteins for analytical purposes, such as high-throughput screening of recombinant enzyme libraries (Olsen *et al.*, 2000) or crystallization studies (Hart & Tarendeau, 2006). Additionally, *E. coli* is becoming increasingly important in whole-cell biocatalysis where it is used as a robust vehicle for the conversion of biochemicals via recombinant enzymes (Wilkinson & Bachmann, 2006).

In most cases, the efficiency of *E. coli* in producing the desired protein is a critical factor in determining its applicability as a host for heterologous protein expression, and optimal conversion of the available substrate into cellular energy and the necessary biosynthetic precursors is paramount. With carbohydrates as the most widely used carbon and energy sources, key metabolic pathways are glycolysis, the pentose phosphate pathway and the tricarboxylic acid (TCA) cycle. Not only are precursor metabolites for all proteinogenic amino acids derived from intermediates of these pathways, but they are also pivotal for energy metabolism; together with the electron transport chain and ATP-synthase. While this central metabolic network is relatively robust (Sauer *et al.*, 1999), it can be perturbed by inadequate nutrient supply, suboptimal physico-chemical conditions or limitations arising from the overexpression of the heterologous protein itself, the result being a reduced energy gain and the loss of valuable carbon through the formation of undesired byproducts. For example, a prevalent problem in cultivations of *E. coli* is the formation of acetate, caused by an imbalance between glycolysis and TCA cycle fluxes (Wolfe, 2005). Fortunately, acetate formation can be overcome in numerous ways (Eiteman & Altman, 2006), one of them being to alter genetically the expression of regulatory genes with an influence on central carbon metabolism, such as *arcA*, whose deletion has recently been shown to lead to a considerable reduction in acetate formation (Vemuri *et al.*, 2006).

One recently discovered gene with the potential to restrict the central carbon metabolism of *E. coli* is the small noncoding RNA (sRNA) gene *ryhB*. First identified in two independent genome-wide sRNA screens (Argaman *et al.*, 2001; Wassarman *et al.*, 2001), it was found to interfere with growth on succinate (Wassarman *et al.*, 2001). Later, *ryhB* was determined to be regulated by the iron uptake regulator Fur and was implicated in the downregulation of numerous genes encoding iron-containing proteins during iron depletion (Massé & Gottesman, 2002). Among the RyhB targets found were transcripts encoding the TCA cycle enzymes

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli strains		
MG1655	F^- λ^- *ilvG rbf*-50 *rph*-1	ATCC: 47076
BO3	MG1655 Δ*ryhB*::Kan^R	This study
BO1	MG1655 Δ*hmp*::Cm^R	This study
BO2	BO1 Δ*ryhB*::Kan^R	This study
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB* $lacI^qZ\Delta M15$ Tn10 (Tet^r)]	Bullock *et al*. (1987)
Plasmids		
pPΔhmp	Cloning plasmid containing flanking regions of *hmp*	Kallio *et al*. (2007)
pACYC177	Cloning plasmid (ori p15A, Amp^R, Kan^R)	GenBank accession: X06402
pACYC184	Cloning plasmid (ori p15A, Tet^R, Cm^R)	GenBank accession: X06403
pKQV4	ori pBR322, P_{tac}, MCS, $lacI^Q$, Amp^R	Strauch *et al*. (1989)
pPPC1	Derivative of pKQV4 containing *vhb*	Kallio *et al*. (1996)
Alpha+GFP	Eukaryotic GFP-expression vector	Crameri *et al*. (1996)
pKQ-gfp	Derivative of pKQV4 containing *gfpuv* from Alpha+GFP	This study

succinate dehydrogenase, fumarase (FumA) and aconitase (AcnA). Meanwhile, global transcriptional analysis of an *E. coli* strain overxpressing RyhB ectopically has revealed that RyhB directly downregulates at least 18 operons, including the second aconitase gene *acnB*, as well as the NADH-dehydrogenase-I-encoding *nuo* operon (Massé *et al*., 2005). These findings suggest a potential for RyhB to shut down the TCA cycle completely and to impede efficient respiration. A previously unanswered question is whether this potential is relevant during recombinant protein production. In principle, RyhB should be obsolete in the presence of sufficient iron, because *ryhB* is tightly regulated, and because RyhB acts in a stoichiometric fashion and is rapidly destroyed upon target interaction (Massé *et al*., 2003). Providing sufficient iron to a culture, however, is not always possible, because iron has an extremely low solubility under aerobic conditions at circumneutral pH (Andrews *et al*., 2003) and calls for the not always practical addition of solubilizing agents.

It was found earlier that constitutive expression of RyhB from a multicopy plasmid severely impairs growth on glucose-containing minimal medium (Wäborg, 2002), and recent findings of others also showed that constitutive RyhB expression in a *fur* mutant has a detrimental effect on growth (Jacques *et al*., 2006). This prompted analysis of the role of RyhB in protein overexpressing *E. coli*. While the influence of RyhB on growth alone has been analyzed by others (Massé & Gottesman, 2002; Jacques *et al*., 2006), this is the first account of RyhB's impact on byproduct formation and the overexpression of heterologous proteins in *E. coli*.

Materials and methods

Bacterial strains, plasmids and DNA manipulations

The bacterial strains and plasmids used in this study are listed in Table 1. Subcloning steps were performed using XL1-Blue. Transformations were carried out as described elsewhere (Chung *et al*., 1989). PCR reactions and other DNA manipulations were performed according to standard procedures (Sambrook & Russell, 2001) and following recommendations of the respective enzymes' suppliers. Plasmid constructs were verified by DNA sequencing.

Construction of plasmid pKQ-gfp

The ORF for the 'GFPuv' variant of green fluorescent protein (GFP) was PCR amplified from the eukaryotic expression vector Alpha+GFP using the primer pair GFE/GRP (Table 2). Digestion of the resulting PCR product with EcoRI–PstI and ligation into EcoRI–PstI-digested pKQV4 yielded pKQ-gfp.

Construction of chromosomal gene replacements

Strain BO1 was derived from MG1655 by replacing the ORF of *hmp* with the chloramphenicol resistance gene (*cat*) from pACYC184. Strains BO2 and BO3 are derivatives of BO1 and MG1655, respectively, in which the chromosomal RyhB-encoding sequence was replaced with the kanamycin-resistance gene (*kpt*) from pACYC177. Putative Rho-independent terminators of both *hmp* and *ryhB* were left in place. All manipulations undertaken to obtain, screen and verify these knock-outs were in accordance with a previous account of the construction of an *hmp* null-mutant (Kallio *et al*., 2007). Selection of Kan^R and Cm^R integrants on Luira–Bertani-Agar plates was performed using kanamycin at 50 μg mL^{-1} and chloramphenicol at 40 μg mL^{-1}, respectively. The PCR primers used in the subcloning of flanking regions and antibiotic resistance genes are listed in Table 2, as are primers used for the verification of the correct insertion of *kpt* and *cat* at the *ryhB* and *hmp* locus, respectively.

Table 2. Oligonucleotides

Name	Sequence* 5′–3′	Restriction site
Subcloning of *gfp*		
GFE	cg*gaattc*ATGGCTAGCAAAGGAGAA	EcoRI
GRP	aaaa*ctgcag*TTATTTGTAGAGCTCATC	PstI
Amplification of *ryhB* flanking regions		
I-LF	atcat*cgagctc*TCGACATATTCACCATTACGC	SacI
I-LR	gg*actagt*TGCATTTGACTCGCATTTG	SpeI
I-RF	gg*actagt*TTAGCCAGCCGGGTGCTG	SpeI
I-RR	cc*gctcgag*TACGGCGTGACGGTCAGC	XhoI
Subcloning of *kpt*		
Kan-F	gg*actagt*CCTGAATCGCCCCATCATCC	SpeI
Kan-R	gg*actagt*CAACAAAGCCGCCGTCCC	SpeI
Verification of *kpt* insertion at the *ryhB* locus		
I-SF	g*ctctaga*TTTCCATCTCAGAAAAAGGGCAC	XbaI
Kan-SR	aaaa*ctgcag*CGCTCGTCATCAAAATCACTCG	PstI
I-SR	aaaa*ctgcag*CGCTGTCAGGAAGCAGGAGTC	PstI
Kan-SF	g*ctctaga*GCCTCTTCCGACCATCAAGC	XbaI
Subcloning of *cat*		
Cm-F	gg*actagt*ACGACCCTGCCCTGAACCG	SpeI
Cm-R	gg*actagt*GCATCACCCGACGCACTTTG	SpeI
Verification of *cat* insertion at the *hmp* locus		
Hmp-SF	g*ctctaga*GGTATCACCTGGTTCCAGCAGC	XbaI
Cm-SR	g*ctctaga*ATCAGCACCTTGTCGCCTTG	XbaI
Hmp-SR	aaaa*ctgcag*CCAAAGGCAACGTCACCCAC	PstI
Cm-SF	aaaa*ctgcag*CCTTTATTCACATTCTTGCCCG	PstI

*Nucleotides homologous to the initial templates are depicted as upper case letters. Lower case letters denote nucleotides added to incorporate restriction sites. Restriction sites are depicted in italic. Primers occupying adjacent rows in the table were used in combination.

Shake flask cultivations

Aerobic shake flask cultures of MG1655: pKQ-gfp and BO3: pKQ-gfp were carried out in baffeled Erlenmeyer flasks of 500 -mL capacity on an Innova 4000 rotatory shaker (New Brunswick Scientific) at 250 r.p.m. and 37 °C. The initial culture volume was 100 mL. Shake flasks were inoculated with 1 mL of a 5 mL preculture, started from a single colony and grown overnight under identical conditions as the corresponding shake flasks.

Media used were either M9 or M63 (Miller, 1972) or MOPS (Neidhardt *et al.*, 1974), prepared by sterile filtration (0.2 μm) and supplemented with glucose (2 g L^{-1}), thiamine (1 mg L^{-1}) and ampicillin (100 mg L^{-1}). All media were prepared using chemicals of analytical grade and high-purity, ultrafiltrated water. Nondisposable vessels were soaked in 2 M HCl for 15 min and then rinsed with high-purity, ultrafiltrated water before use in order to remove all traces of iron.

GFP expression was induced by adding Isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM when the $OD_{600\,nm}$ (OD_{600}) reached a value of 0.4.

Microaerobic fed-batch cultivations

Microaerobic fed-batch cultivations of strains BO1:pPPC1 and BO2:pPPC1 were carried out essentially as described previously (Bollinger *et al.*, 2001), except that complex media components (yeast extract and casamino acids) were omitted.

Physiological analysis

Growth was monitored by measuring $OD_{600\,nm}$ with a Novaspec II spectrophotometer (LKB Pharmacia). Cell dry weight (CDW) values were determined for each culture individually and in triplicate from culture samples of a known $OD_{600\,nm}$ as follows: 10 mL of culture broth was dispensed into a washed, dried and preweighed glass centrifuge tube. Cells were pelleted by centrifugation at 4000 ***g***, 4 °C for 10 min, washed once with water and pelleted again before being dried, first at 80 °C for 24 h and then to a constant weight *in vacuo*. The dried samples were then weighed in order to determine the correlation between $OD_{600\,nm}$ and CDW.

The glucose and acetate levels in culture supernatants were determined enzymatically in a Beckman SYNCHRON CX5CE autoanalyzer, using commercial kits available from Beckman for glucose and Scil Diagnostics (Viernheim, Germany) for acetate. Organic acid concentrations in the supernatant from fed-batch cultures were determined via HPLC as described elsewhere (Fuhrer *et al.*, 2005). Exhaust gases (O_2 and CO_2) from bioreactor cultivations were

monitored with a Brüel & Kjær Emission Monitor Type 3427. For shake flask batch cultures, specific growth (μ), glucose consumption (q_{Glc}) and acetate production (q_{Ac}) rates as well as biomass yield on glucose ($Y_{x/Glc}$) were determined by linear regression analysis from data collected during the exponential growth phase. For fed-batch cultures, respective yields were determined by dividing net amounts of biomass, byproduct or CO_2 formed by the net amount of glucose consumed during the time period between zero and 30 h postinoculation.

Preparation of protein extracts

Cells from 40 mL of culture broth were harvested from shake flask and biorector cultivations by centrifugation at 4000 ***g***, 4 °C for 10 min. Cells were resuspended in an appropriate amount of lysis buffer (100 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA) and were disrupted by passing them twice through an AMINCO French Pressure Cell (SLM Instruments Inc., Urbana, IL) at 1000 psi. The resulting lysate was cleared from cell debris by two sequential centrifugation steps at 15 000 ***g***, 4 °C for 20 min.

The total protein concentration in soluble cell extracts was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

GFP assay

The fluorescence of GFPuv was recorded at 509 nm in soluble cell extracts with a Shimadzu RF-5001PC spectrofluorometer, using an excitation wavelength of 395 nm. Lysis buffer was used to determine background fluorescence. Relative fluorescence intensities were normalized according to the samples' total protein content.

CO-binding assay

Carbon monoxide difference spectra of VHb-containing cell extracts from microaerobic bioreactor cultures, treated as described previously (Webster & Liu, 1974), were recorded with a Perkin-Elmer Lambda2 spectrometer between 400 and 500 nm. VHb concentrations in the samples were calculated from peak and trough absorption values using the absorption difference coefficient of $1.067 \times 10^5\ M^{-1}\ cm^{-1}$ (Hart *et al*., 1994). In order to allow for a comparison between samples, measurements were normalized according to the samples' total protein content.

Results and discussion

Shake flask experiments

Wild-type MG1655 as well as the *ryhB* knock-out mutant BO3 were transformed with the medium-copy-number plasmid pKQ-gfp, designed for IPTG-inducible overexpression of GFP. Comparative growth experiments in shake flasks were carried out in three different minimal media in order to assess the effect of RyhB on growth, biomass yield, glucose consumption, acetate formation and GFP expression levels. The media used were M9, M63 and MOPS with glucose as the sole carbon source. These media were chosen because they are the most widely used chemically defined media for growing *E. coli*; the outcome of the experiments thus being of broader interest. Also, the three media contain incremental amounts of iron: the M9 recipe relies solely on adventitious iron from impurities in other media components, and M63 has a mere 1 μM of $FeSO_4$ added. These concentrations are rather low and, indeed, *E. coli* has previously been shown to express RyhB during exponential

Table 3. Physiology of aerobic batch cultures

Strain	μ* (h^{-1})	$Y_{X/Glc}$* [mg (CDW) g^{-1}]	q_{Glc}* [mmol g^{-1} (CDW) h^{-1}]	q_{Ac}* [mmol g^{-1} (CDW) h^{-1}]	GFP content* [RFU mg^{-1} (total protein)]
M9 medium					
MG1655	0.56 ± 0.03	365 ± 35	7.27 ± 1.41	8.13 ± 0.85	33 ± 3
BO3	0.56 ± 0.07	425 ± 56	7.26 ± 1.45	4.42 ± 0.22	18 ± 3
Change†	0%	+16%	0%	**−46%**	**−45%**
M63 medium					
MG1655	0.62 ± 0.02	501 ± 20	6.64 ± 0.41	4.61 ± 0.15	25 ± 2
BO3	0.66 ± 0.01	539 ± 46	6.57 ± 0.60	3.64 ± 0.20	16 ± 1
Change†	+6%	+8%	−1%	**−21%**	**−37%**
MOPS medium					
MG1655	0.71 ± 0.02	514 ± 42	7.11 ± 0.12	2.54 ± 0.14	37 ± 2
BO3	0.71 ± 0.01	532 ± 20	7.04 ± 0.35	2.16 ± 0.68	40 ± 4
Change†	0%	+3%	−1%	−15%	9%

*Indicated values are means ± SD from at least three independent cultivations.

†Relative change in mean value effected by deletion of *ryhB*. The mean values determined for the *ryhB*$^+$ strain were assumed as 100%. Bold values indicate differences that are statistically significant at the 95% confidence level, as determined by unpaired Student's *t*-test.

growth on M9 (Argaman *et al.*, 2001) and M63 (Wassarman *et al.*, 2001). MOPS medium, on the contrary, is a well-balanced medium whose compostition has been modeled to follow the elementary composition of an *E. coli* cell (Neidhardt *et al.*, 1974). It contains 10 μM of $FeSO_4$, which was expected to be sufficient to prevent derepression of *ryhB*, rendering an *ryhB* mutant phenotypically silent.

The results of the shake flask cultures are presented in Table 3. Consistent with previous reports (Jacques *et al.*, 2006), lack of *ryhB* was unable to elicit a significant change in growth rate or biomass yield in any of the media tested. Jacques et al (2006) found an *ryhB*$^-$ strain to exhibit a prolonged lag-phase under iron-deplete conditions. While obtaining this effect with a plasmid-free strain required pretreatment of the medium with an appropriate iron chelator, it is imaginable that plasmid replication may lead to a stress sufficient for an *ryhB* knock-out to show this prolonged lag-phase phenotype even when iron is present. As can be seen from Fig. 1, however, none of the mutant cultures took longer to commence growth than its *ryhB*$^+$ counterpart, which shows that iron requirements are not accentuated by the presence of a pBR322-derived plasmid. Glucose uptake rates were also unaffected by the state of *ryhB*, regardless of the medium used. Acetate formation rates, on the other hand, were considerably influenced, with significant reductions in specific acetate productivity seen in M9 and M63, but not in MOPS medium (see Table 3). Also, the extent to which acetate output declined in the absence of *ryhB* was stronger in M9 than in M63. These findings suggest that the effects of RyhB on central carbon metabolism are dependent on extracellular iron, with a threshold value lying somewhere between what can be provided by M63 and MOPS medium, respectively.

Rather surprisingly, the wild-type strain had a higher GFP content than its RyhB-free counterpart in M9 and M63 media. With growth rate, biomass yield and glucose uptake rate being equal in both strains, the fact that the *ryhB*$^+$ strain produced, at the same time, more acetate and more protein suggests that neither carbon nor energy availability is limiting GFP expression. Apparently, insufficient iron supply has a specific effect on plasmid-based protein expression but does not affect the general fitness of the cell. As it seems this effect is attenuated by RyhB, however, it remains to be analyzed what exactly the role of RyhB is in this attenuation.

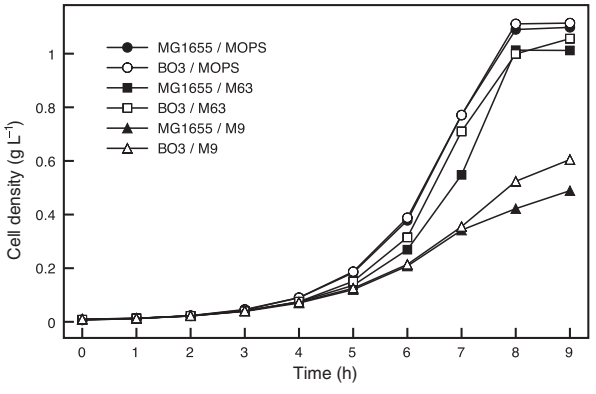

Fig. 1. Growth trajectories from aerobic batch cultivations of strains MG1655 (*ryhB*$^+$) and BO3 (Δ*ryhB*::Kan^R), expressing GFP from the multicopy plasmid pKQ-gfp. Indicated values for each time point are means from three independent shake-flask cultivations.

It should be noted, that the changes effected by knocking out *ryhB* are only of a relative nature. The highest absolute values for growth rate, biomass yield and GFP content, as well as the lowest absolute values for acetate output were found in the non-iron-limiting MOPS medium (Table 3). Therefore, the role of RyhB in plasmid-based overexpression of a heterologous protein will most likely remain of academic interest only as long as sufficient iron can be supplied through the medium.

Microaerobic fed-batch cultures

While iron depletion arising from insufficient extracellular supplies can be overcome by altering media composition, there is another potential cause for the derepression of the *ryhB* gene that cannot be avoided easily: if the rate of synthesis of a heterologous iron-containing protein is near-exhausting for the cellular iron uptake machinery, it seems possible that free intracellular Fe^{2+} can become insufficient for Fur to exhibit significant DNA-binding activity. This would then lead to constitutive or at least prolonged expression of RyhB and the aforementioned negative consequences.

Examples where recombinant expression of iron-containing proteins in *E. coli* is of biotechnological relevance include the high-level production of cytochrome P450-type enzymes (Barnes, 1996) or the use of the heterologous bacterial hemoglobin VHb from *Vitreoscilla* as an auxiliary protein to enhance growth and productivity under conditions where adequate oxygen supply cannot be guaranteed (Frey & Kallio, 2003). Intriguingly, metabolic flux analysis has previously revealed a downregulation of TCA cycle flux (Tsai *et al.*, 1996) or even a complete interruption thereof (Frey *et al.*, 2001) upon overexpression of VHb in *E. coli* under microaerobic conditions, and it seems plausible to assume that RyhB could be the reason for these findings.

In order to analyze whether RyhB plays a role in VHb-overexpressing *E. coli* and whether the beneficial effects of VHb could be enhanced even further by averting a potential downregulation of the TCA cycle by RyhB, an *ryhB* knock-out strain, capable of overexpressing VHb, was constructed. To avoid interference from the endogenous hemoglobin Hmp, whose expression has been reported before as being induced upon iron limitation (Poole *et al.*, 1996), the

hmp::Cm^R mutant BO1 was constructed first and the *hmp*::Cm^R *ryhB*::Kan^R double-mutant BO2 was derived from it.

Strains BO1 and BO2, both harboring pPPC1, were grown in microaerobic fed-batch cultivations where, at first glance, the state of the *ryhB* gene did not seem to have any impact. As depicted in Fig. 2, both strains grew equally well. A closer analysis, however, showed that the *ryhB*$^-$ strain diverted substantially less carbon towards the net formation of acetate and that it did so despite having a markedly lower intracellular VHb content (see Table 4). This is surprising, because both microaerobic growth as well as fermentative byproduct formation have previously been shown to respond to VHb expression in a dose-dependent manner (Tsai *et al.*, 1996): Tsai and colleagues showed that under virtually the same growth conditions as those used here, biomass accumulation in a wild-type K-12 strain was directly proportional to VHb content, while the formation of fermentative byproducts, including acetate, correlated inversely with intracellular VHb. Clearly, the *ryhB* mutant is better suited for microaerobic growth than its *ryhB*-positive counterpart, because it was capable of growing equally well despite producing less VHb. Further investigations into the physiological consequences of knocking out *ryhB* were undertaken to determine the reason for this superiority: measurements of organic acid byproducts commonly produced by *E. coli* during mixed acid fermentation revealed no statistically significant differences between the two strains with respect to the percentages of carbon they converted to pyruvate (2–3%), to (DL)-lactate (20–22%), to formate (4–6%) and to succinate (below 1%). As a contrast, offgas analysis of the bioreactors showed that the *ryhB* mutant produced a significantly higher amount of CO_2 per unit glucose consumed (see Table 4). This strongly suggests that the *ryhB* knock-out strain benefits from a greater carbon flux through the TCA cycle and the higher ATP formation rate associated with that flux. An attempt to establish a carbon balance failed to trace approximately equal amounts of carbon consumed for both strains (12%). This is most likely attributable to the formation of the only common fermentative byproduct that was not measured, namely ethanol, and indicates that the only relevant changes in central carbon metabolism effected by the knock-out of *ryhB* are those concerning acetate and CO_2 formation.

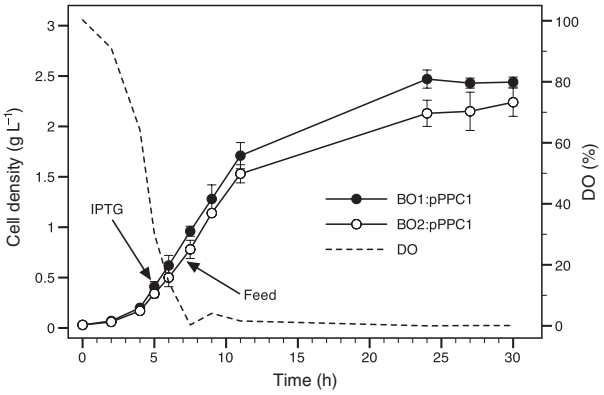

Fig. 2. Growth trajectories from microaerobic fed-batch cultivations of strains BO1 (*ryhB*$^+$) and BO2 (Δ*ryhB*::Kan^R), expressing heterologous hemoglobin VHb from the multicopy plasmid pPPC1. Indicated values for each time point are means ± SD from three independent bioreactor cultivations. For dissolved oxygen (DO), a representative curve from one of the cultures is included, variations between the individual cultures being only marginal. Indicated DO values are denoted as percent of air saturation of the bioreactors. Arrows indicate the time points when VHb expression was induced (IPTG) and when feeding mode was started (Feed), respectively.

What remains elusive is the cause for the lower VHb concentration in the *ryhB* mutant. One possible explanation is that in the wild type, the iron-sparing effect of RyhB allowed additional iron to be incorporated into heme and, consequently, VHb, while in the mutant, this extra iron was incorporated into RyhB target proteins. However, it is also possible that the lower VHb expression observed here is due to the same effect as the lower GFP expression in the shake flask cultures of BO3:pKQ-gfp.

General conclusions

This study was carried out with the aim of analyzing the influence of RyhB on the physiology of plasmid-bearing, protein-overproducing *E. coli* and the possibility of ameliorating, by means of knocking out the *ryhB* gene, specific protein expression during iron limitation. While growth

Table 4. Physiology of microaerobic fed-batch cultures

Strain	Final CDW* (g L^{-1})	$Y_{X/Glc}$* [mg (CDW) g^{-1}]	$Y_{Ac/Glc}$* (% carbon)	$Y_{CO2/Glc}$* (% carbon)	VHb content* [nmol mg^{-1} (total protein)]
BO1:pPPC1 (*ryhB*+)	2.44 ± 0.04	61 ± 2	24 ± 3	29 ± 1	2.3 ± 0.5
BO2:pPPC1 (Δ*ryhB*)	2.24 ± 0.14	55 ± 3	17 ± 1	39 ± 3	1.3 ± 0.2
Change†	− 8%	− 9%	**− 29%**	**+34%**	**− 43%**

*Indicated values are means ± SD from three independent cultivations.

†Relative change effected by deletion of *ryhB*. The values for the *ryhB*$^+$ strain were assumed as 100%. Bold values indicate differences that are statistically significant at the 95% confidence level, as determined by unpaired Student's *t*-test.

remained unaffected by the state of *ryhB*, much in accordance with previous findings (Jacques *et al*., 2006), it could clearly be shown that knocking out *ryhB* leads to a considerable reduction in acetate output during apparent iron limitation. This finding is significant, because it indicates that iron limitation can have a serious impact on central carbon metabolism and should therefore be avoided whenever possible.

Another important discovery is the fact that an *ryhB*$^{+}$ and an *ryhB*$^{-}$ strain respond differently to the overexpression of VHb. This clearly shows the ability of an iron-containing protein to lead to *ryhB*-inducing conditions even in the presence of ample extracellular iron (200 μM Fe^{3+}, supplied as iron-citrate). This is an important caveat for the overexpression of iron-containing proteins for biotechnological purposes.

Acknowledgements

The authors are indebted to Heidi Ernst and Martina Röthlisberger for DNA sequencing and to Paula Kühnis for assistance with the cultivations.

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