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Life cycle analysis of the model organism *Rhodopirellula baltica* SH 1^T by transcriptome studies

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Summary

The marine organism *Rhodopirellula baltica* is a representative of the globally distributed phylum *Planctomycetes* whose members exhibit an intriguing lifestyle and cell morphology. The analysis of *R. baltica*'s genome has revealed many biotechnologically promising features including a set of unique sulfatases and C1-metabolism genes. Salt resistance and the potential for adhesion in the adult phase of the cell cycle were observed during cultivation. To promote the understanding of this model organism and to specify the functions of potentially useful genes, gene expression throughout a growth curve was monitored using a whole genome microarray approach. Transcriptional profiling suggests that a large number of hypothetical proteins are active within the cell cycle and in the formation of the different cell morphologies. Numerous genes with potential biotechnological applications were found to be differentially regulated, revealing further characteristics of their functions and regulation mechanisms. More specifically, the experiments shed light on the expression patterns of genes belonging to the organism's general stress response, those involved in the reorganization of its genome and those effecting morphological changes. These transcriptomic results contribute to a better understanding of thus far unknown molecular elements of cell biology. Further, they pave the way for the biotechnological exploitation of *R. baltica*'s distinctive metabolic features as a step towards sourcing the phylum *Planctomycetes* at large.

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Introduction

Rhodopirellula baltica (RB) SH 1^T was isolated from the water column in the Kiel Fjord (Baltic Sea) and taxonomically grouped within the bacterial phylum *Planctomycetes* (Schlesner *et al*., 2004). Members of this group are abundant in aquatic habitats and are considered to play a significant role in carbon cycling (Glöckner *et al*., 2003). *Planctomycetes* share several unique properties, such as peptidoglycan-free proteinaceous cell walls (Liesack *et al*., 1986), intracellular compartmentalization (Lindsay *et al*., 2001) and a mode of reproduction via budding. The latter trait results in a life cycle comprised of motile and sessile morphotypes and resembles that of *Caulobacter crescentus* (Gade *et al*., 2005a). The annotation of the completely sequenced genome of *R. baltica* allowed an initial assessment of its genetic potential and revealed several interesting and surprising traits such as a high number of sulfatase genes, a fascinating set of carbohydrate-active enzymes [CAZymes (Cantarel *et al*., 2009), (Michel *et al*., 2006; Dabin *et al*., 2008)] and a conspicuous C1-metabolism pathway (Glöckner *et al*. 2003 and references therein). Moreover, several features were detected that promise a potential for biotechnological exploitation. For instance, the genome harbours enzymes for the synthesis of complex organic molecules with possible applications in the pharmaceutical field, such as a gene set encoding a polyketide synthase, and enzymes important for the production of natural products in the food or animal-feed industry, such as those active in vitamin and amino acid biosynthesis. The application of sulfatases of *R. baltica* for the stereoselective conversion of racemates has been discussed in Wagner and Horn (2006) and Gadler and colleagues (2006). Many of the genes are unique and may be employed to enhance the efficiency of microbial strains already in biotechnical use. For example, genes coding for antibiotic resistance might be used to immunize production strains against certain

antibiotics, while genes conferring virulence resistance could improve the robustness of these strains. *Rhodopirellula baltica* is capable of a sessile lifestyle, a feature which is desirable for cell recovery during the production of secreted products in biotechnological processes. Salt resistance is desirable for production strains as industrial media or waste water often have high salt concentrations. The fact that about half of the genes in *R. baltica* currently lack an assigned function further demonstrates the huge, but so far hidden, genetic potential of this model organism.

During the life cycle of *R. baltica*, cells change their morphology, form swarmer cells to sessile cells with holdfast substances, produce secondary metabolites and experience different conditions such as nutrient excess and deprivation as well as high cell densities. The analysis of growth-dependent expression patterns during the life cycle of *R. baltica* enables a screening for genes for specific physiological functions, morphotype differentiation and cellular processes most probably typical for this phylum.

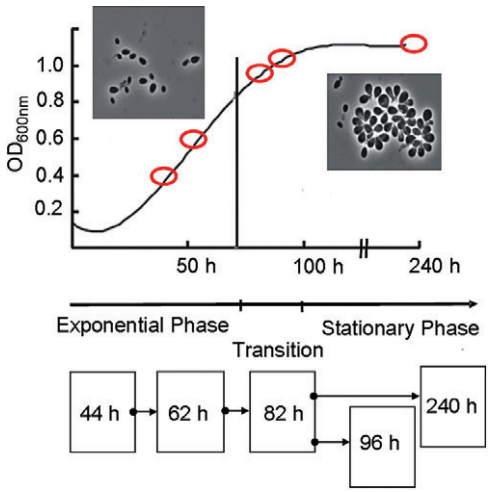

Fig. 1. Schematic drawing of the life cycle of *R. baltica*, modified after (Gade *et al.*, 2005a) and hybridization scheme: *R. baltica* cultures were harvested at five time points indicated by the red circles. cDNA samples were hybridized in two replicates. Dots and arrowheads represent samples labelled with Alexa 547 and Alexa 647 respectively.

Results and discussion

Gene expression studies

Rhodopirellula baltica was grown in a defined mineral medium with glucose as a sole carbon source to study changes in gene expression that reflect the organism's life cycle phases and response to nutrient depletion. The growth of *R. baltica* cells could not be synchronized. Nevertheless, microscopic examination showed that the culture is dominated by swarmer and budding cells in the early exponential growth phase, shifting to single and budding cells as well as rosettes in the transition phase, while the stationary phase is dominated by rosette formations. These observations are in accordance with previous results (Gade *et al.*, 2005a).

Global differential gene expression results

Cultures of *R. baltica* were harvested at the onset of the exponential phase (after 44 h of incubation), during the exponential phase (mid-log phase, 62 h), in the transition phase between exponential and stationary phase (late-log phase, 82 h), and in the early and late stationary phase (96 h and 240 h respectively). Figure 1 presents an overview of the system in which the different growth phases were compared. The total number of genes detected as differentially regulated for each of the growth stage investigated is shown in Table 1. A complete list of genes can be found in the Appendix S1. Samples taken from the early log-phase (44 h) and the exponential phase (62 h) were differentiated by the up- and downregulation of 149 genes, of which 56% have been annotated as hypothetical proteins. The comparison between the exponential (62 h) and transition phase (82 h) revealed the regulation of only 90 genes, 40% of which have unknown functionality. A pronounced change in the transcriptome, with the differential regulation of 235 genes (59% hypothetical proteins), was observed when the transition phase (82 h) was compared with the stationary phase (96 h). A comparison between cultures from 82 h with those of late stationary phase (240 h) revealed the most pronounced changes of the transcriptome in our study: 863 genes were differentially expressed (Fig. 2), of which 58% are annotated as hypothetical proteins.

Table 1. Total number of regulated genes during each measured time point of the growth curve and the fraction of the regulated genes annotated as conserved and/or hypothetical.

Number of regulated genes	62 h vs. 44 h	82 h vs. 62 h	96 h vs. 82 h	240 h vs. 82 h
Total (%)*	149 (2%)	90 (1%)	235 (3%)	863 (12%)
Encoding hypothetical proteins (%)**	84 (56%)	40 (44%)	139 (59%)	499 (58%)

(%)* relative to the total number of 7325 open reading frames (ORFs) annotated in *R. baltica* genome.
(%)** relative to the total number of regulated genes.

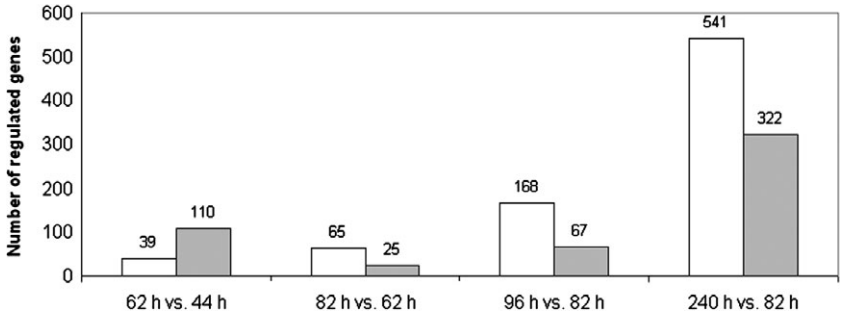

Fig. 2. Number of significantly up- and downregulated genes during the growth curve at the four time points which were compared. White means upregulated and grey downregulated.

The analysis of the exponential phase (a comparison of the early-log at 44 h and late-log at 82 h with mid-log at 62 h) showed that a maximum fraction of 2% of the total number of genes was regulated. This reflects the favourable nutritional conditions encountered throughout these growth stages (Gade *et al*., 2005a).

The assignment of differentially expressed genes to functional cluster of orthologous group (COG) classes offered a first impression of *R. baltica*'s metabolic activity through the different growth stages (Table 2). Genes associated with the 'metabolism of amino acids' [E] and 'carbohydrates' [G], genes for 'energy production and conservation' [C], as well as genes for 'DNA replication and recombination' [L] were downregulated in mid-exponential phase (62 h) in comparison with the 44 h sample. The repression (62 h vs. 44 h) of genes coding for oxidases (RB4416 and RB5701), a peptidase [RB4269, which was also found regulated under stress conditions (Wecker *et al*., 2009)], a synthase (RB6443) as well as lipases and esterases (RB7072 and RB3413) suggests a lower metabolic activity in the cells most likely due to lower nutrient availability compared with the early exponential phase.

Interestingly, genes of the 'cell envelope biogenesis, outer membrane' [M] and 'cell motility and secretion' [N] categories, such as genes coding for flagellin [RB4454;

Table 2. Number of regulated genes with an assigned COG-category compared with the whole genome functional classification assignment according to the NCBI database (cut-off e-value e-4).

		62 h vs. 44 h		82 h vs. 62 h		96 h vs. 82 h		240 h vs. 82 h	
COG-class	Total in genome	Up	Down	Up	Down	Up	Down	Up	Down
C	170	0	4	0	1	5	0	9	7
D	18	0	0	0	0	0	0	0	0
E	211	1	3	2	1	1	1	7	6
F	64	0	0	0	0	1	0	1	1
G	172	1	2	2	0	4	0	8	5
H	117	0	1	0	0	1	0	11	0
I	75	0	1	0	0	0	0	1	2
J	144	0	1	0	0	4	0	4	9
K	156	0	1	1	1	2	0	5	10
L	176	0	2	1	0	0	0	13	4
M	142	0	3	1	0	2	0	8	6
N	102	0	3	0	0	1	0	1	1
O	106	0	0	9	0	6	0	3	12
P	234	0	4	1	1	3	1	15	6
Q	141	0	2	2	0	4	0	8	4
R	418	0	8	3	1	13	2	19	11
S	177	1	2	2	0	5	2	11	10
T	210	0	0	2	0	2	0	6	6

Rows: [C] Energy production and conversion, [D] Cell division and chromosome partitioning, [E] Amino acid transport and metabolism, [F] Nucleotide transport and metabolism, [G] Carbohydrate transport and metabolism, [H] Coenzyme metabolism, [I] Lipid metabolism, [J] Translation, ribosomal structure and biogenesis, [K] Transcription, [L] DNA replication, recombination and repair, [M] Cell envelope biogenesis, outer membrane, [N] Cell motility and secretion, [O] Posttranslational modification, protein turnover, chaperones, [P] Inorganic ion transport and metabolism, [Q] Secondary metabolites biosynthesis, transport and catabolism, [R] General function prediction only, [S] Function unknown, [T] Signal transduction mechanisms.

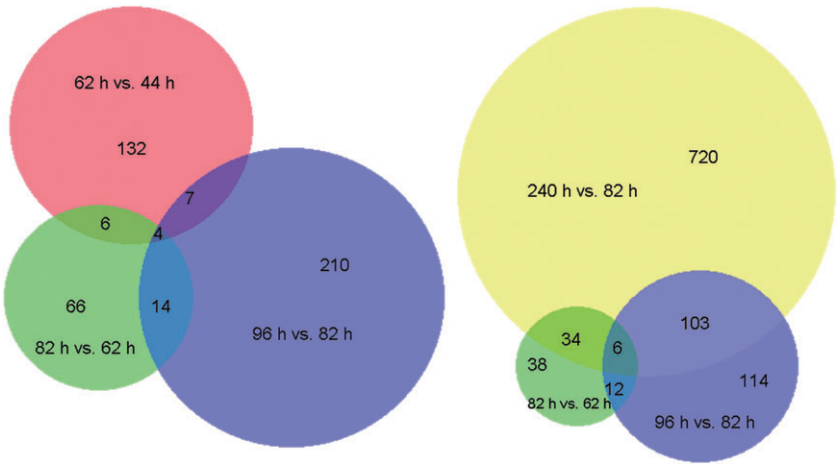

Fig. 3. Venn diagrams showing the distribution of all regulated genes.

also downregulated under stress (Wecker *et al*., 2009)], bacterial type II and III secretion system protein (RB5174) and for diverse transferases (RB6305 and RB6624), were repressed at 62 h. Thus, when compared with the mid-exponential phase (62 h), it appears that the onset of the exponential phase (44 h) triggers a remodelling of the cell wall and morphology, perhaps explained by an enhancement of the swarmer cell production. This response is likely to be very expensive energetically, and would explain the higher expression level of genes from COG category C observed (Jolly *et al*., 2007).

Comparison of the transition phase and stationary growth phase (82 h) with the mid-exponential phase (62 h) revealed that more genes were regulated in the stationary phase than the mid-exponential. It could be speculated that genes necessary for exponential growth under favourable conditions were already expressed in early log and mid-log phase (44 h and 62 h). Still, several genes showed differential expression. For example, *R. baltica* increased the glutamate dehydrogenase (RB6930) level, thus confirming proteome experiments (Hieu *et al*., 2008). The enzyme is involved in the biosynthesis of arginine, glutamate and proline. The latter is one of the major components of the *R. baltica's* cell wall. Upregulation of glutamate dehydrogenase could indicate that the organism's cell wall composition has been adapted to more unfavourable conditions in response to the decreasing nutrient concentration.

The cell density reached its highest point during the transition phase of the *R. baltica* flask culture. Stressed cells need to adapt metabolically to the nutrient limitation in the culture, which is reflected by our results. Genes coding for glutathione peroxidase (RB2244), thioredoxin (RB12160, see also Hieu *et al*., 2008) and bacterioferritin comigratory protein (RB12362), universal stress protein (*usp*E, RB4742) and chaperones (e.g. RB8966) were induced and diverse dehydrogenases, hydrolases and reductases were up- and downregulated for metabolic adaptation and to prepare for long-term survival under unfavourable conditions. The regulation of RB2244, RB12362, RB8966 is in line with the proteome results of the life cycle (Gade *et al*., 2005a).

Earlier experiments have shown that *R. baltica* survives for at least 14 days in the stationary phase at 28°C (Gade *et al*., 2005a). We compared expression profiles of the early (96 h) and the late stationary phase (240 h) with that of the transition phase (82 h). The overlap of both sample sets (96 h vs. 82 h and 240 h vs. 82 h), visualized by a Venn diagram (Fig. 3), revealed 103 genes with similar regulation behaviour. Seventy-one out of the 103 genes are annotated as hypothetical proteins and most of the remaining 32 genes are stress-related, as expected.

The general response of *R. baltica* cells to the stationary phase environment includes the induction of genes associated with energy production, amino acid biosynthesis, signal transduction, transcriptional regulation, stress response and protein folding. Genes coding for the enzymes of carbon metabolism, translation control, energy production and amino acid biosynthesis were repressed. *Rhodopirellula baltica* decreased the expression level of genes belonging to the ribosomal machinery (RB12197, RB2543, RB264, RB5801, RB5804, RB7022, RB7818, RB8253, RB8725 and RB9304), transcription regulation (RB10339, RB10458, RB11223, RB12372 and RB1392) and DNA replication and recombination (RB11863 and RB3281) in the late stationary phase, probably due to reduced growth activity. Additionally, the organism expressed many genes coding for transposases, integrases and recombinases (RB10096, RB11303, RB11750, RB1190, RB3144, RB4826, RB5887, RB7388, RB12239, RB2186, RB6736, RB9907,

RB6167, RB7389 and RB934), suggesting that under stress conditions, or in the late stationary phase, genome rearrangements take place. Due to the scarcity of operon structures in the genome of *R. baltica*, the rearrangement might be necessary to enable an efficient transcription during stressful phases. Six out of the 15 genes (RB11750, RB12239, RB2186, RB9907, RB7389 and RB934) have been found active in response to non-physiological conditions of temperature and salinity (Wecker *et al*., 2009), which indicates that expression of these genes constitutes a response to general stress. The upregulation of genes for the phenylalanine, tyrosine and tryptophane biosynthesis (RB6822 and RB6147) is noteworthy. This is consistent with proteome data (Gade *et al*., 2005a), but the physiological meaning is currently unknown. Furthermore, genes involved in the biosynthesis of serine, threonine and glycine (RB6932) and of lysine (RB4382) were upregulated.

Reaction to oxygen depletion is shown as part of the stress response as well. It is known from *Agrobacter tumefaciens* and *Rhodobacter sphaeroides* that the lack of oxygen triggers the increased production of ubiquinone (Cluis *et al*., 2007). The same effect can be seen in our results with the induction of genes for the ubiquinone biosynthesis (RB2748, RB2749 and RB2750) in the stationary phase. Also, it seems that the composition of the cell wall was modified in response to changes in the physiological state of the *R. baltica* cell during the late stationary phase. The organism exported more polysaccharides, as shown by the enhanced formation of rosettes indicating production of a holdfast substance. Diverse genes belonging to the cell membrane class [M] were upregulated. *Rhodopirellula baltica* activated the production of many outer membrane transporters and biopolymers (RB12053, RB12055, RB9308, RB1120, RB754), as well as several transferases (RB12690, RB2507, RB2985, RB5846) for alteration of the lipopolysaccharides that belong to the cell membrane. RB12053, RB12055 and RB12690 showed also a response to stressful conditions in our earlier study (Wecker *et al*., 2009). In addition, we found 19 genes annotated as membrane proteins to be regulated, as well as 13 secreted proteins. Two genes coding for bacterial microcompartment proteins (RB2585 and RB2586) were upregulated. These proteins supposedly form primitive organelles in a variety of bacteria (Kerfeld *et al*., 2005). Such findings may indicate that the cell wall composition was adapted to maintain the cell shape. It could be that after entering the stationary phase, *R. baltica* cells, similar to what was found for the marine bacterium '*Candidatus* Pelagibacter ubique', undergo morphological changes (cell shape), enabling them to return to exponential growth after being transferred to fresh medium or to a nutrient-rich environment (Sowell *et al*., 2008).

Cell division and cell cycle

Little is known about the cell division and replication regulation in *Planctomycetes*. None of the 18 *R. baltica* genes assigned to the COG category 'cell division and chromosome partitioning' [D] were found to be regulated during this study, nor were any of the unclassified COG homologues of genes that are generally known to be involved in the cell division. This suggests that in *R. baltica* a number of regulated genes with thus far unknown function must play a role in the regulation of replication and cell division. However, clues can be garnered from the fact that *R. baltica* exhibits a life cycle similar to *C. crescentus*. DnaK/DnaJ and GroES/GroEL are the best-characterized chaperone systems in bacteria and are induced in cell cycle regulation and under heat shock in *C. crescentus* (Susin *et al*., 2006). Those chaperones have an important role in chromosome replication and partition and/or cell division. The *R. baltica* genome harbours genes coding for *groEL* (RB10629 and RB8970), *dnaK* (RB5754 and RB5755) and *dnaJ* [RB8972 (Wecker *et al*., 2009)] in the genome. Of these, only *groEL* (RB10629) is upregulated at 82 h (transition phase) in the life cycle experiment, which could also indicate a general response to stress (Wecker *et al*., 2009). In addition, both *groEL*-genes showed a regulation during the proteome experiments (Gade *et al*., 2005a). *Rhodopirellula baltica* also possesses two genes encoding DnaA (RB11579 and RB1706), a protein which initiates not only the DNA replication, but also promotes the expression of the components necessary for successful chromosome duplication in *C. crescentus*. However, *dnaA* was not found to be regulated in our experiments.

In order to acquire more knowledge about genes possibly involved in changes of the morphotype throughout the cell cycle and the role of genes classified as hypothetical proteins, the gene expression data were clustered (see *Experimental procedures*). Clusters can be found in Table 3 and Appendix S2. The clusters 4, 12 and 17 group genes which are only regulated in the exponential phase where nutrient limitation or stress conditions are unlikely. Therefore, these genes can be supposed to have a function in the morphology differentiation to swarmer cells or in the budding process. Genes belonging to clusters 11, 13, 19, 20, 21, 22 and 23 are upregulated during the transition phase. Genes annotated as hypothetical proteins belonging to the early stationary phase (96 h) were grouped in clusters 18, 24 and 25. Cluster 18 and 24 consist of many genes annotated as hypothetical proteins, which might be involved in morphology changes, e.g. cell shape and wall. Finally, cluster 14 genes are upregulated in the exponential (62 h) and downregulated in the stationary (96 h) phase in comparison with the transition phase. This cluster contains one sulfatase gene and 14 genes encoding hypothetical proteins. It can be

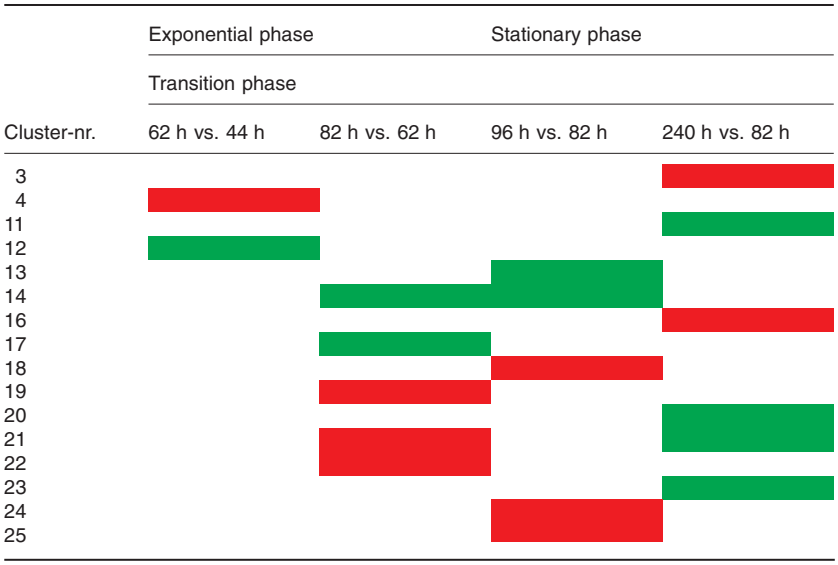

Table 3. Overview of the k-means clustering of the different growth stages of *R. baltica*.

The expression average of the relevant clusters (row) is shown in colour over the compared growth stages (columns). Red = induced genes and green = repressed genes.

assumed that these have a function in the morphotype differentiation.

Lipids

In this study, delta-9 desaturase (RB4876), which belongs to the unsaturated fatty acid biosynthesis pathway, was upregulated in the transition phase (82 h). Furthermore, expression of malonyl-CoA acyl carrier protein transacylase (RB314), an enzyme involved in fatty acid biosynthesis, was upregulated in the late stationary phase (240 h). Results from proteome studies (Gade *et al*., 2005a) indicate that the biosynthesis of sulfated saturated fatty acids occurs specifically during the stationary phase, but this could not be reproduced on the transcriptome level. In our recent study (Wecker *et al*., 2009), RB314 (together with the acyl carrier protein, RB318) was also upregulated under stress-inducing conditions. The increased expression of these genes might be part of a general stress response, possibly to enhance the stability of the membrane to protect the cell.

Tetrahydromethanopterin-(H4MPT)-linked C1-compound metabolism

Rhodopirellula baltica has the genetic potential to degrade C1-compounds, and the functional annotation predicted all enzymes necessary for the oxidation of formaldehyde to formate (Glöckner *et al*., 2003). How this pathway benefits the organism in its environment is so far unknown. Out of the 19 proposed genes encoding enzymes of C1-metabolism (Woebken *et al*., 2007), four genes were regulated in our experiment: *fae* (RB9338), *mtdA* (EC 1.5.1.5, RB10295) and *orf7* (RB10246) were induced in the late stationary phase (240 h) compared with the transition phase (82 h), whereas *orfY* (RB10299) encoding a potential ATP-dependent carboligase (COG2232) was induced in the exponential and repressed in the early stationary phase. Table 4 gives an overview on C1-genes that have, to our knowledge, been found expressed so far. Although *in silico* studies show the presence of the C1-pathway in other *Planctomycetes* (Chistoserdova *et al*., 2004; Woebken *et al*., 2007), the specific functions of the genes in *Planctomycetes* are still unknown. However, the expression of some genes observed in this study suggests that this pathway has physiological relevance in *R. baltica* and most likely in other *Planctomycetes* as well.

Potential biotechnologically relevant genes

Throughout the life cycle, biotechnologically interesting genes were found to be regulated. The microarray experiments provide valuable information under which conditions these genes are expressed and how they interact. With a doubling time of approximately 12 h and complex growth conditions, *R. baltica* has limitations as a production strain. Nevertheless, the respective genes could be cloned and heterologously expressed. Considered here are gene products relevant for (i) new biotechnological processes, (ii) the improvement of existing processes, (iii) processes which could help to shift classical chemical production towards enzyme catalysed systems and (iv) genes with potentially medical applications. An overview of the regulated genes organized according to their possible application is presented in Appendix S3.

Table 4. Overview of the expressed or regulated genes coding for the C1-transfer enzymes in *R. baltica*.

		Transcriptome		Proteome
ID	Gene name or product	Current study	Woebken *et al*., 2007	Gade *et al*., 2005b
RB6210	Tetrahydromethanopterin biosynthesis protein (ORF21)		×	
RB6300	pabA			×
RB6759	mch			×
RB8806	ptpS			
RB9338	fae	1.5	×	
RB9834	fm(w)dA		×	
RB9836	fm(w)dC		×	
RB10246	ORF7	2.6	×	
RB10295	mtdA	1.7	×	
RB10299	ORFY	1.7/ 2.5/ –1.7/ 0		
RB11969	Protein containing ATP-grasp fold, DUF201-type (ORF1)		×	

Food industry and animal feeding form a globally important market and many of the processes existing today leave room for improvements. Almost 50 genes of *R. baltica*, which were found to be differentially expressed in the present study, have potential applications in this field. Among them are genes for vitamin and sugar synthesis or amino acid conversion and several of the organism's sulfatase genes. The efficient and inexpensive synthesis of amino acids and vitamins is increasingly important as feed additives to raise livestock for a growing world population and sulfatases can be used in the food industry as thickening agent, and to convert racemates to single stereoisomers that may play a role in the pharmaceutical field.

The 41 genes listed in the category 'pharmaceutical & medical application' have a potential to improve current biosynthesis methods for vitamins, antibacterial drugs or complex organic molecules synthesis, with applications in cancer research. The biotechnological production of certain amino acids is important for the pharmaceutical industry. An example would be the biosynthesis of tryptophan, which can be administered as a mild sleeping drug (Hartmann, 1977). An additional instance of a gene product that may be valuable for the development of new medical treatments is an FKBP-like isomerase found to be differentially expressed in *R. baltica*. FKBP isomerases are involved in the immunoregulation.

Other enzymes, such as polymerases, might improve methods in molecular biology and would therefore benefit medical research or analytical techniques. For example, nine polymerase-associated genes are regulated in this study.

In the emerging field of white biotechnology, *R. baltica* could offer genes to improve the robustness of existing production strains with respect to antibiotics and phage resistance, for the production of washing detergents, for the synthesis of natural substances or for waste water treatment. The ability of *R. baltica* to form a holdfast substance might prove valuable as well. In many biotechnology processes the biomass is harvested together with the desired product and thus lost, which leads to additional costs for replacing the production cells.

Hence, it is favourable to retain the biomass during harvesting secreted proteins or enzymes, for example, by employing attached cells.

Sulfatases

The genome of *R. baltica* harbours an exceptional number of 110 sulfatase genes. Based on sequence similarity, it has been proposed that they are involved in the utilization of carbon from complex sulfated heteropolysaccharides (Glöckner *et al*., 2003).

During transcriptional analysis of the different *R. baltica* growth stages, we found 12 sulfatases up- or downregulated including one putative choline sulfatase (RB2254), three different arylsulfatases (RB4815, RB684 and RB686), two N-acetylgalactosamine-6-sulfate sulfatase (RB13148, RB5195), one mucin-desulfating sulfatase and four other sulfatases (RB110, RB10612, RB5294 and RB5424) (Table 5). Organic sulfate is not likely to have caused the induction of the sulfatase genes observed in this study as the medium lacked sulfated carbon. Five of the sulfatase genes mentioned above (RB110, RB2254, RB4815, RB5294 and RB686) were also found upregulated when the expression profile of *R. baltica* cells grown on a solid surface was compared with that of cells grown in liquid culture (data not shown), and RB2254 was found in the extracellular proteome (data not shown). The observation that certain sulfatases were only expressed in specific growth phases suggests that degrading polymers is not the only role of sulfatase genes in *R. baltica*. Based on the differential expression pattern of these sulfatase genes we propose an involvement in structural remodelling during the morphological differentiation of *R. baltica* cells, such as the formation of the holdfast substance, which is needed to attach to surfaces, the production of

Table 5. Differentially expressed sulfatase genes of *R. baltica* during the growth curve plus additional regulation data.

ID	Product	AA	Signalp	Gene name	EC number	62 vs. 44 h	62 vs. 82 h	96 vs. 82 h	240 vs. 82 h	Remarks
RB10612	Sulfatase family protein	492	0						1.9	
RB110	Sulfatase family protein	582	0			–1.5				W
RB13148	N-acetyl-galactoseamine-6-sulfate sulfatase	1012	0.2				–1.5	–2.9	1.5	T
RB2254	Choline sulfatase	541	0	betC	3.1.6.6				1.8	W, P
RB4815	Arylsulfatase A	491	1	arsA	3.1.6.8				1.9	W
RB5195	N-acetyl-galactoseamine-6-sulfate sulfatase	505	1	arsb	3.1.6.12				1.5	
RB5282	Mucin-desulfating sulfatase	578	0.5	betC	3.1.6.6				–1.7	
RB5294	Sulfatase	533	0						1.6	W, T
RB5424	Sulfatase	573	0			–1.8				
RB684	Arylsulfatase precursor	653	0.8	atsA	3.1.6.1				–1.5	T
RB686	Arylsulfatase	549	1	atsA	3.1.6.1	–1.7				W, M
RB7875	Arylsulfatase A precursor	492	0.9	arsA	3.1.6.8				2.3	

W: transcriptome studies. cells attached to chemostat wall (unpublished data), M: metagenome studies (Woebken *et al.*, 2007), P: extracellular proteome (Hieu *et al.*, 2008), T: transcriptome data (Wecker *et al.*, 2009).

slime polysaccharides (Kumar, 2007) or in the cell wall formation, which consists of a protein sacculus with disulfide bonds (Liesack *et al.*, 1986).

Another hypothesis is that, when sensing decreasing glucose concentrations, the cells express sulfatases to prepare for the degradation of alternative carbon sources. More experimental work is needed to clarify the diverse roles of the unusually high number of sulfatase genes in *R. baltica*.

Carbohydrate-active enzymes

Carbohydrate-active enzymes are responsible for the synthesis (glycosyltransferases), the modification (carbohydrate esterases) and the degradation [glycoside hydrolases (GH) and polysaccharide lyases] of polysaccharides and glycoconjugates, which play an important role in, e.g. cell wall synthesis and carbon storage. The degradation of carbohydrates taps a special nutrient source for the microorganisms (Michel *et al.*, 2006). As the predicted lifestyle of *R. baltica* in its natural environment involves the attachment to and degradation of marine snow, its CAZymes might have a function in both the formation of the hold-fast substance and the breakdown of marine snow particles. Similarly, the field of application in biotechnology comprises the degradation of complex substances as well as the synthesis of specific organic substances for pharmaceutical use or for food industry.

Throughout *R. baltica*'s life cycle, four CAZymes were found to be regulated. RB8895 is a sialidase, which is upregulated during exponential growth and downregulated in the stationary phase. Sialic acids are particularly abundant in animals in extracellular glycoproteins and glycolipids. When present in bacteria, sialic acids are found mostly in capsular polysaccharides and lipopolysaccharides (Angata and Varki, 2002). However, the key enzymes for sialic acid biosynthesis, N-acetyl neuraminate synthase (NeuB, EC 2.5.1.56) and N-acylneuraminate cytidyltransferase (NeuA, EC 2.7.7.43) are absent in the genome of *R. baltica*. In contrast, *R. baltica* possesses a complete pathway for sialic acid catabolism with seven family GH33 sialidases (including RB8895), an N-acetylneuraminate lyase (RB3352, EC 4.1.3.3), which was found to be expressed on the proteome level (Gade *et al.*, 2005c), and a N-acetyl-D-glucosamine 2 epimerase (RB3348, EC 5.1.3.8). In absence of endogenous sialic acids, the role of RB8895 in the cell cycle is rather intriguing. The other three regulated genes encoding CAZymes (RB548, RB4894, RB5196) all belong to the family GH13, but have distinct activities. These enzymes are induced in the early or late stationary phase. RB5196 is highly similar to the amylosucrase from *Neisseria polysaccharea* (52% sequence identity) (Büttcher *et al.*, 1997). This enzyme is not a GH, but a transglycosidase which catalyses the synthesis of an

alpha-(1,4)-linked glucan polymer from sucrose. Therefore an alpha-(1,4) glucan is likely produced during the *R. baltica* cell cycle. Such a marine amylosucrase has exciting biotechnological potential, as it uses sucrose as sugar donor instead of an expensive activated sugar, such as ADP- or UDP-glucose. RB548 and RB4894 are both related to glycogen metabolism, encoding a glycogen branching and debranching enzyme respectively. RB548 was shown to be active on the proteome level (Gade *et al*., 2005c). A systematic search of glycogen-related enzymes (Ball and Morell, 2003) demonstrated that *R. baltica* possesses a complete glycogen metabolism (Dabin *et al*. in preparation). Therefore, it can be assumed that *R. baltica* makes use of its intracellular carbon storage (glycogen) in order to produce other polysaccharides or glycoconjugates, for example, to generate the holdfast substance. RB548 was also found to be expressed in proteome studies of cells grown on agar plates (Gade *et al*., 2005a).

Hypothetical proteins co-regulated with genes of potential biotechnologically relevant applications

Given the limited functional knowledge on cellular processes in *Planctomycetes,* it was not surprising that a large number of genes found to be differentially expressed in this study encoded (conserved) hypothetical proteins. In order to get information on the potential function of those genes, we delineated groups of genes with similar expression patterns during culture growth. Information on the genes populating clusters, which comprise genes with possible functions in biotechnology, can be found in the Appendix S2. Table 3 shows the average expression pattern of the clusters, in which genes with potential biotechnological applications were found.

We first focused on genes with unknown functions, which were co-regulated with potentially biotechnologically relevant genes (Appendix S4). For example, cluster 16 (repression in stationary phase) contains genes upregulated in the stationary and late stationary phase, among them several sulfatases and genes involved in amino acid turn over, as well as almost 150 genes with unknown function. Cluster 3 (induction in late stationary phase) includes genes that play a potential role in the formation of the holdfast substance. So far, little is known about the holdfast substance of *R. baltica* and thus the 185 genes of unknown function could serve as a starting point to learn more about the organisms' ability to attach. Three sulfatases were also assigned to cluster 3 (induction in late stationary phase), emphasizing the proposal of an additional function which is connected with the cell structure formation.

Interestingly, four of the nine polymerase-associated genes in cluster 11 (repression in late stationary phase) were gathered together with 67 hypothetical proteins. The possibility that some of these genes have a similar function should be further investigated.

Several genes involved in amino acid or sugar metabolism in cluster 20 (induced in late stationary phase) are co-regulated with 103 hypothetical proteins. These hypothetical proteins may be candidates for the completion of the amino acid and sugar metabolism pathways of *R. baltica*. The co-regulated hypothetical genes are hence prime targets to detect and confirm alternative ways of biochemical conversions in the laboratory.

Conclusions

In this transcriptome study, gene regulation throughout the life cycle of *R. baltica* was examined in order to learn more about this model organism's behaviour as well as its regulation of genes with putative biotechnological application.

This study documents transcriptomic changes concomitant with morphological changes during the exponential growth of an *R. baltica* culture. The lack of nutrients and a high cell density in the stationary phase imposes the activation of general stress response genes, genes for the reorganization of the genome and the cell wall, and possibly also for the formation of the holdfast substance. The results suggest that a subset of the 110 sulfatases may be involved in the formation of planctomycete-specific morphological features (e.g. cell wall components or holdfast substance), rather than in the degradation of sulfated carbon compounds.

Proteins of unknown function constitute a major part of the regulated genes in all growth stages of *R. baltica.* Their correlation to potential biotechnologically relevant genes, their expression at specific growth stages and their correspondence with certain morphotypes provide some preliminary insights into their function. In the future, next generation sequencing technologies will be applied to confirm the results presented here and to complement the data with information about regulatory RNA.

Our results provide a substantial foundation for further studies on the transcriptomic and metabolomic elucidation of the unknown mechanisms behind the lifestyle, cell cycle and cell division of *R. baltica.* Furthermore, the insights they provide form a basis for more directed experiments.

Experimental procedures

Cultivation of Rhodopirellula baltica *cells*

Cells of *R. baltica* were grown in batch cultures (500 ml) with mineral medium containing 10 mM glucose as sole carbon and 1 mM ammonium chloride as nitrogen source (Rabus *et al*., 2002). The *R. baltica* cultures were incubated at 28°C

on a rotary shaker. Two independent batch cultures (biological replicates) per time point were inoculated and were treated separately over the whole experimental processes.

Preparation of samples for hybridizations and microarray processing

Cultures were harvested independently by centrifugation (Beckman Coulter™ Avanti™ J-20XP, JA10 Rotor, 20 min, 6000 r.p.m., 4°C), after different incubation times (44, 62, 82, 96 and 240 h) for expression profiling and microscopic analysis. The culture broth was collected in 500 ml of tubes and swirled briefly in an ethanol/dry ice bath to rapidly cool down the cultures and 'freeze' the RNA profile. Subsequently, the broth was centrifuged at 6000 r.p.m. for 20 min at 4°C (Beckman Coulter™ Avanti™ J-20XP, JA10 Rotor). The pellets were re-suspended in 0.1 M Tris-HCl and then re-centrifuged to obtain cell pellets that were subsequently shock-frozen in liquid nitrogen and stored at −80°C. Total RNA isolation and cDNA synthesis were performed as previously described (Wecker *et al.*, 2009). For hybridization at least 2 μg of Alexa 547 dye-labeled (PlatinumBright: Nucleic Acid Labelling Kit – 547 Red/Orange, Kreatech, Amsterdam, the Netherlands) and 2 μg of Alexa 647 dye-labelled total cDNA (PlatinumBright: Nucleic Acid Labelling Kit – 647 Infrared, Kreatech, Amsterdam, the Netherlands) were combined and taken up in a final volume of 100 μl of DIG Easy Hyb hybridization solution (Roche Diagnostics, Mannheim, Germany). After the blocking step, the sample solution was applied to the arrays, denatured at 95°C for 3 min and hybridized under stringent conditions at 52°C for over 12 h. After hybridization, slides were washed at room temperature in ULTRArray Low Stringency Wash Buffer (Ambion, Austin, USA) and dried by N_2.

In two independent hybridizations cDNA samples were hybridized according to Fig. 1. Dots and arrowheads represent samples labelled with Alexa 547 and Alexa 647 respectively. The data shown are based on the analysis of the two technical and biological replicates performed for each of the conditions.

For the microarrays a set of oligonucleotides corresponding to the whole genome of *R. baltica* SH 1^T (Pirellula AROS Version 1.0) was purchased from Operon (Cologne, Germany) and diluted to 20 μm concentration in Micro Spotting Solution Plus spotting buffer (Telechem Sunnyvale, CA, USA). Spotting was done in three replicates onto GAPS II aminosilane slides (Corning, Schiphol-Rijk, the Netherlands) using a SpotArray 24 spotting device (Perkin Elmer, Wellesley, MA, USA). Post-processing and blocking of the slides were done according to the manufacturer's instructions. Blocking, hybridization and washing were carried out in an automated hybridization station HS400 (Tecan, Crailsheim, Germany). A more detailed description of the array can be found at the NCBI's Gene Expression Omnibus database (GEO, 2009) under accession Platform number GPL7654 and in Wecker and colleagues (2009).

Signal detection and data analysis

Slides were scanned at a resolution of 5 μm using a ScanArray Express Microarray scanner (Perkin Elmer). The image analysis software provided with this scanner was used for automatic spot detection and signal quantification. Raw data were automatically processed using the microarray data analysis software tool MADA (MADA, 2010). Series of probe replicates were tested for outliers using the outlier test implemented in MADA. First of all, only signals were considered as positive if mean spot pixel intensity was higher than local mean background pixel intensity plus twice the standard deviation of the local background. Only those spot signals were further processed, for which all three replicates, which are present for each gene on the whole genome microarray, were considered as a valid, positive signal. An outlier test was performed for the three replicates. In this procedure, the standard deviation for all replicates is calculated. Next, one of the replicates is left out and the standard deviation is computed once more. In case the newly calculated standard deviation differs more than 50% from the former one, the replicate is flagged as an outlier and removed from the data set. This procedure is rerun for all replicates. Subsequently, the mean of the remaining replicates is computed. Expression is described through the ratio (R) and intensity (I), where R is the fluorescence log ratio of one experimental time point over the other [e.g. R = log2 (result of channel for sample 44 h/result of channel for sample 62 h)] and I is the log mean fluorescence intensity [e.g. I = log10 (result of channel 44 h × result of channel 62 h)].

LOWESS normalization was applied on an R-versus-I plot with a smoothing factor of 0.5. The expression data (ratio) of all hybridizations (array replicates) were averaged and combined to one expression data point (ratio).

An expression ratio greater than 1.5 or less than −1.5 was used as a threshold for significant changes. The threshold was determined in a self-self-hybridization experiment. Reference sample material was labelled with both dyes and hybridized onto the same array. All steps were carried out as described above. The resulting signals were analysed and the log ratio was calculated. In theory, all log ratios should be zero in this experimental set up. However, due to noise the log ratios usually differ from zero. The cut-off was determined in an R-versus-I plot (data not shown).

Cluster analysis and database

All genes of the genome and their gene expression values were clustered using the k-means clustering approach [Euclidean distance metric, k = 25 clusters and nine iterations (max. 500)] with the software tool Multi-experiment Viewer MeV Version 4.0.2 from the TM4 microarray software suite (Saeed *et al.*, 2003). The genome of *R. baltica* was automatically re-annotated based on updated homology searches [June 2005 – MicHanThi (Quast, 2006)]. The updated annotation including all tool results are publicly available (Quast, 2009). JCoast (Richter *et al.*, 2008) was used as a tool for the visualization, interpretation, statistic (COG-assignment) and comparison of genomic data stored in GenDB V2.2 (Meyer *et al.*, 2003). Venn diagrams were generated by BioVenn (Hulsen *et al.*, 2008).

A detailed description of the expression data is available from the GEO database under the accession Series number GSE19405.

Competing interests

The authors declare that they have no competing interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. List of differentially expressed genes.
Appendix S2. List of selected clusters out of the k-means clustering.
Appendix S3. List of regulated genes with potential applications in biotechnology.
Appendix S4. List of genes with unknown function, which are co-regulated with genes of potential biotechnological application.

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