Karlsruher Institut für Technologie

MOLECULAR CHARACTERISATION OF CULTIVATION PROCESSES

– STRESS-INDUCED RHAMNOLIPID PRODUCTION –

zur Erlangung des akademischen Grades eines

DOKTORS DER INGENIEURWISSENSCHAFTEN (Dr.-Ing.)

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genehmigte

DISSERTATION

von

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To my family and Philippe

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I. Abstract

The application of external stress on bacteria is a promising approach to evoke a targeted cellular response in bioprocesses, such as the synthesis of secondary metabolites to improve production capacities. A prime example is the biotechnological production of rhamnolipids, a biosurfactant that are used in diverse technical, industrial and care products and can serve as an eco-friendly alternative to the widely-used synthetic surfactants Rhamnolipids are naturally produced by the Gram-negative, opportunistic human pathogen *Pseudomonas aeruginosa* during stationary growth phase. However, a lack of understanding regarding metabolic and regulatory events and relatively low product yields are the major drawbacks of industrial production. The regulation of rhamnolipid synthesis is tightly governed by a complex regulatory network including bacterial quorum sensing (QS) systems, different sigma factors as well as additional regulatory factors, which are dependent on many factors, such as cell density or nutrient limitations.

In this study, gene expression profiles of various genes that have been associated with the regulation of rhamnolipid synthesis as well as genes for rhamnolipid synthesis itself are presented to identify important points of regulation during such bio processes.

The underlying method for gene expression analyses in this thesis, quantitative Polymerase Chain Reaction (qPCR), was established first. Besides careful primer design, evaluation of PCR efficiency for each primer pair is an important prerequisite before employing qPCR for gene activity measurements. In total, 23 genes were selected and tested for correct PCR efficiency to create a 'molecular toolbox'. Ensuing from successful method establishment, qPCR was applied to demonstrate its potential to adequately and reliantly analyse differential gene expression during multi-scale bioprocesses. Various different cultivation scenarios were examined in small-scale shake flask experiments to investigate dependencies on external stress factors, such as cell density, nutrient limitation or nitrogen source. For this purpose, rhamnolipid synthesis was also assessed on production level by measuring the amount of mono- and dirhamnolipids in the culture supernatant using liquid chromatography tandem mass spectrometry

(LC/MS/MS). Additionally, gfp reporter gene assays were developed and employed to study the time-dependent course of expression of the two synthesis genes.

Most promising amongst different approaches was the omission of iron from the culture medium. Both expression of rhamnolipid synthesis genes, as well as rhamnolipid production was increased under iron depleting cultivation conditions. Furthermore, a cell density-dependent increase in rhamnolipid synthesis gene expression could be demonstrated. Based upon the positive results obtained under iron depletion, a strategy for the controlled and targeted induction of iron limiting conditions and hence rhamnolipid production was developed. The bio process might be directly and purposely transferred from growth stage after sufficient biomass formation into production stage after sequestration of iron at any time during the cultivation by specific iron chelating compounds. For this purpose, six different chelators were used to induce iron limiting conditions in small-scale shake flask experiments. However, the hypothesis remains to be hypothetical.

For large-scale rhamnolipid production, *P. aeruginosa* PAO1 was grown under nitrogen limitation with sunflower oil as carbon and nitrate as nitrogen source in a 42 L-bioreactor using a production medium designated as 'standard' by Müller et al. 2010. Gene expression profiles were assessed using qPCR over the entire time course of the cultivation in order to be able to identify possible regulatory bottlenecks. Based upon the obtained expression data, a first working hypothesis could be established for the drastic decline in the expression of the gene for monorhamnolipid synthesis, *rhlA,* by the end of the cultivation process.

Following the small-scale shake flask experiments using iron limiting cultivation conditions, a scale-up was initiated in a 2.2 L-bioreactor using the same *P. aeruginosa* strain grown in iron-free production medium. However, the positive effect observed in the small-scale shake flask experiments could not be achieved for the larger-scale set-up. Such up-scaling problems are often encountered during process development.

The molecular toolbox certainly might help to identify crucial regulatory bottlenecks, which hinder to achieve the same production potential observed for small-scale

cultivations. Differences in gene activities could be observed between the two cultivations that might help to explain possible up-scaling issues.

II. Zusammenfassung

Externer Stress induziert bei Bakterien eine molekulare Antwort, welche gezielt eingesetzt werden könnte, um die Synthese bestimmter Stoffe in Bioprozessen zu aktivieren und die Produktionskapazität zu verbessern. Ein Musterbeispiel ist die biotechnologische Produktion von Biotensiden, sogenannte Rhamnolipide, welche in einer Vielzahl technischer, industrieller und Pflegeprodukten Verwendung finden und als eine umweltfreundliche Alternative zu den weitverbreiteten synthetischen Tensiden herangezogen werden können. Rhamnolipide werden natürlicherweise von dem Gram-negativen, opportunistischen Humanpathogen *Pseudomonas aeruginosa* in der stationären Wachstumsphase gebildet. Die bisher größten Hindernisse der industriellen Produktion sind neben mangelnder Kenntnisse über metabolische und regulatorische Ereignisse, die relativ niedrigen Produktionsausbeuten. Die Regulation der Rhamnolipidsynthese unterliegt einer strengen Kontrolle durch ein komplexes Regulationsnetzwerk, woran bakterielle Quorum Sensing Systeme, sowie verschiedene Sigmafaktoren und zusätzliche Regulationsfaktoren beteiligt sind. Diese sind abhängig von einer Vielzahl äußerer Faktoren, wie Zelldichte oder Nährstofflimitierung.

In dieser Studie werden Expressionsprofile verschiedener Gene präsentiert, welche mit der Rhamnolipidsynthese und ihrer Regulation assoziiert sind, um wichtige Regulationspunkte während Bioprozessen zu identifizieren.

Zuerst wurde die grundlegende Methode zur Analyse der Genexpression, quantitative Polymerase Kettenreaktion (quantitative Polymerase Chain Reaction (qPCR)) entwickelt. Die wichtigsten Voraussetzungen, um die qPCR für die Messung von Genaktivitäten einzusetzen, sind neben einem sorgfältigen Primerdesign, die Bestimmung der PCR-Effizienz für jedes Primerpaar. Insgesamt wurden so 23 Gene ausgewählt und auf geeignete PCR-Effizienz überprüft, so dass eine „molekulare Werkzeugbox“ entwickelt werden konnte. Diese wurde anschließend erfolgreich eingesetzt, um ihr Potential zur adäquaten und zuverlässigen Analyse differentieller Genexpression während multi-skaliger Bioprozesse zu demonstrieren. Verschiedene Kultivierungsszenarien wurden daher in kleinvolumigen Schüttelkolbenversuchen untersucht, um die Abhängigkeit der Rhamnolipidsynthese von externen Stressfaktoren,

wie Zelldichte, Nährstofflimitierung oder Stickstoffquelle, zu ermitteln. Zusätzlich wurde der Gehalt an Mono- und Di-Rhamnolipiden chromatographisch im Kulturüberstand bestimmt, um die molekularbiologischen Daten zu bestätigen. Des Weiteren wurden Gfp-Reportergen-Assays für die beiden Synthesegene entwickelt und eingesetzt, um den zeitlichen Verlauf der Expression der beiden Synthesegene zu untersuchen.

Sowohl die Expression der beiden Rhamnolipidsynthesegene, als auch die Rhamnolipidproduktion waren unter Eisen-limitierenden Kultivierungsbedingungen erhöht. Am vielversprechendsten zeigte sich das Weglassen von Eisen aus dem Kulturmedium. Des Weiteren konnte eine Zelldichte-abhängige Erhöhung der Rhamnolipidsynthesegene gezeigt werden. Basierend auf diesen positiven Ergebnissen, die unter Eisen-limitierenden Bedingungen beobachtet wurden, wurde eine Strategie zur kontrollierten und gezielten Induktion von Eisenmangelbedingungen und damit einhergehender Rhamnolipidproduktion entwickelt. Mithilfe von spezifischen Eisen-komplexierenden Chelatoren könnte der Bioprozess gezielt durch das Abfangen von Eisen von der Wachstums- in die Produktionsphase überführt werden. Dazu wurden sechs verschiedene Chelatoren untersucht, um Eisen-limitierende Bedingungen in Schüttelkolbenversuchen zu induzieren. Diese Hypothese konnte allerdings nicht bestätigt werden.

Für die großvolumige Rhamnolipidproduktion wurde *P. aeruginosa* PAO1 unter Stickstofflimitierung mit Sonnenblumenöl als Kohlenstoff- und Nitrat als Stickstoffquelle in einem 42-Liter Bioreaktor kultiviert. Die Zusammensetzung des Produktionsmediums wird als „Standardbedingung“ bezeichnet, beschrieben von Müller et al. 2010. Genexpressionsprofile mittels qPCR wurden während des gesamten Kultivierungszeitraums erstellt, um mögliche regulatorische Engpässe zu identifizieren. Basierend auf den molekularbiologischen Daten wurde eine erste Arbeitshypothese erstellt, anhand derer sich der drastische Abfall der Expression des Gens für die Monorhamnolipidsynthese, *rhlA*, zum Ende des Prozesses, erklären lässt. So konnte der „Standardprozess“ erfolgreich molekularbiologisch charakterisiert werden.

Analog zu den kleinvolumigen Schüttelkolbenversuchen unter Eisen-limitierenden Bedingungen wurde der gleiche *P. aeruginosa*-Stamm in einem 2,2-Liter Bioreaktor in Eisen-freiem Produktionsmedium kultiviert. Allerdings konnte der positive Effekt für

den großvolumigen Scale-Up-Versuch nicht erreicht werden. Solche Up-Scaling-Probleme werden in der Prozessentwicklung häufig beobachtet.

Die entwickelte „molekulare Werkzeugbox“ könnte dabei helfen, entscheidende regulatorische Engpässe zu identifizieren, die einen Transfer von klein- nach großvolumigen Reaktoren erschweren. So konnten Unterschiede in den Genaktivitäten zwischen den verschieden großen Kultivierungen aufgezeigt werden, die erste Hinweise zur Lösung möglicher Up-Scaling-Probleme liefern könnten.

III. List of Publications and Presentations

i. Original Papers and Reviews

2013 **A Schmidberger**, M Henkel, R Hausmann and T Schwartz

"Expression of Genes involved in Rhamnolipid Synthesis in *Pseudomonas aeruginosa* PAO1 in a Bioreactor Cultivation"
Journal of Applied Microbiology and Biotechnology
(DOI 10.1007/s00253-013-4891-0)

2013 M Henkel, **A Schmidberger**, C Kühnert, J Beuker, T Bernard, T Schwartz, C Syldatk, and R Hausmann
"Kinetic Modeling of the Time Course of *N*-Butyryl-Homoserine Lactone Concentration during Batch Cultivations of *Pseudomonas aeruginosa* PAO1"
Journal of Applied Microbiology and Biotechnology
(DOI 10.1007/s00253-013-5024-5)

2014 **A Schmidberger**, M Henkel, R Hausmann, and T Schwartz
"Influence of Ferric Iron on Gene Expression and Rhamnolipid Synthesis during Batch Cultivation of *Pseudomonas aeruginosa* PAO1"
Journal of Applied Microbiology and Biotechnology
(DOI 10.1007/s00253-014-5747-y)

2014 M Henkel, **A Schmidberger**, M Vogelbacher, C Kühnert, J Beuker, T Bernard, T Schwartz, C Syldatk, and R Hausmann
"Kinetic Modeling of Rhamnolipid Production of *Pseudomonas aeruginosa* PAO1 including Cell-density Dependent Regulation"
Journal of Applied Microbiology and Biotechnology
(DOI 10.1007/s00253-014-5750-3)

ii. Talks and Presentations

March 2012 **Poster Presentation** "Molecular Mechanisms of Rhamnolipid Synthesis in *Pseudomonas aeruginosa* during Batch Cultivation"

Annual Conference of the Association for General and Applied Microbiology (VAAM), Tübingen

September 2012 **Poster Presentation**

"ProcessNet" - Annual Conference of the Dechema e.V., Karlsruhe

March 2013 **Talk** „Iron-dependent Regulation of Rhamnolipid Synthesis in *Pseudomonas aeruginosa* PAO1"

Annual Conference of the Association for General and Applied Microbiology (VAAM), Bremen

April 2013 **Poster Presentation** „Iron-dependent Regulation of Rhamnolipid Synthesis in *Pseudomonas aeruginosa* PAO1"

Poster Presentation *"*Model-based Optimization of Rhamnolipid Biosurfactant Production by *Pseudomonas aeruginosa"*

European Congress of Applied Biotechnology (ECAB)/ European Congress of Chemical Engineering (ECCE), The Hague, Netherlands

May 2013 **Poster Presentation** „Iron-dependent Regulation of Rhamnolipid Synthesis in *Pseudomonas aeruginosa* PAO1"

"Biosurfactants" – Conference of the Dechema e.V., Frankfurt (Main)

May 2014 **Poster Presentation** „Influence of Iron Depletion on Multi-scale Rhamnolipid Production in *Pseudomonas aeruginosa* PAO1"

"Biomaterials – Made in Bioreactors" Conference of the Dechema e.V., Radebeul

IV. Theoretical Background

i. Objective

There is a growing demand for economic bioprocesses based on sustainable resources rather than petrochemical-derived substances. Particular attention has been paid to rhamnolipids - surface active glycolipids - that are naturally produced by *Pseudomonas aeruginosa*. Rhamnolipids have gained increased attention over the past years due to their versatile chemical and biological properties as well as numerous biotechnological applications. However, rhamnolipid synthesis is tightly governed by a complex growth-dependent regulatory network, which is the major drawback besides low product yields, expensive downstream processing and excessive foaming. Quantitative comprehension of the molecular and metabolic mechanisms during bioprocesses is therefore key to manipulating and improving rhamnolipid production capacities in *P. aeruginosa*.

ii. Aim of the Work

In this doctoral study, gene expression profiles of various genes that have been associated with the regulation of rhamnolipid synthesis as well as genes for rhamnolipid synthesis itself are presented. The method of choice for generating expression profiles was quantitative Real-Time PCR, which allows accurate and reliable assessment of gene expression.

To begin with, the primer systems for gene expression analyses were selected from the *P. aeruginosa* regulatory network described by current literature (Fig. 1a). The selected primers for reference and target genes were then evaluated for their PCR efficiency. This primer repertoire was subsequently tested by a system validation approach to yield the 'molecular toolbox' for process optimisation (Fig. 1b). Batch cultivations were performed by the Technical Biology of the Karlsruhe Institute of Technology and samples were obtained, RNA was isolated and cDNA generated by reverse transcription, which was subsequently used for Real-Time PCR to yield relative gene expression data. Based upon this data, process manipulation strategies could be developed.

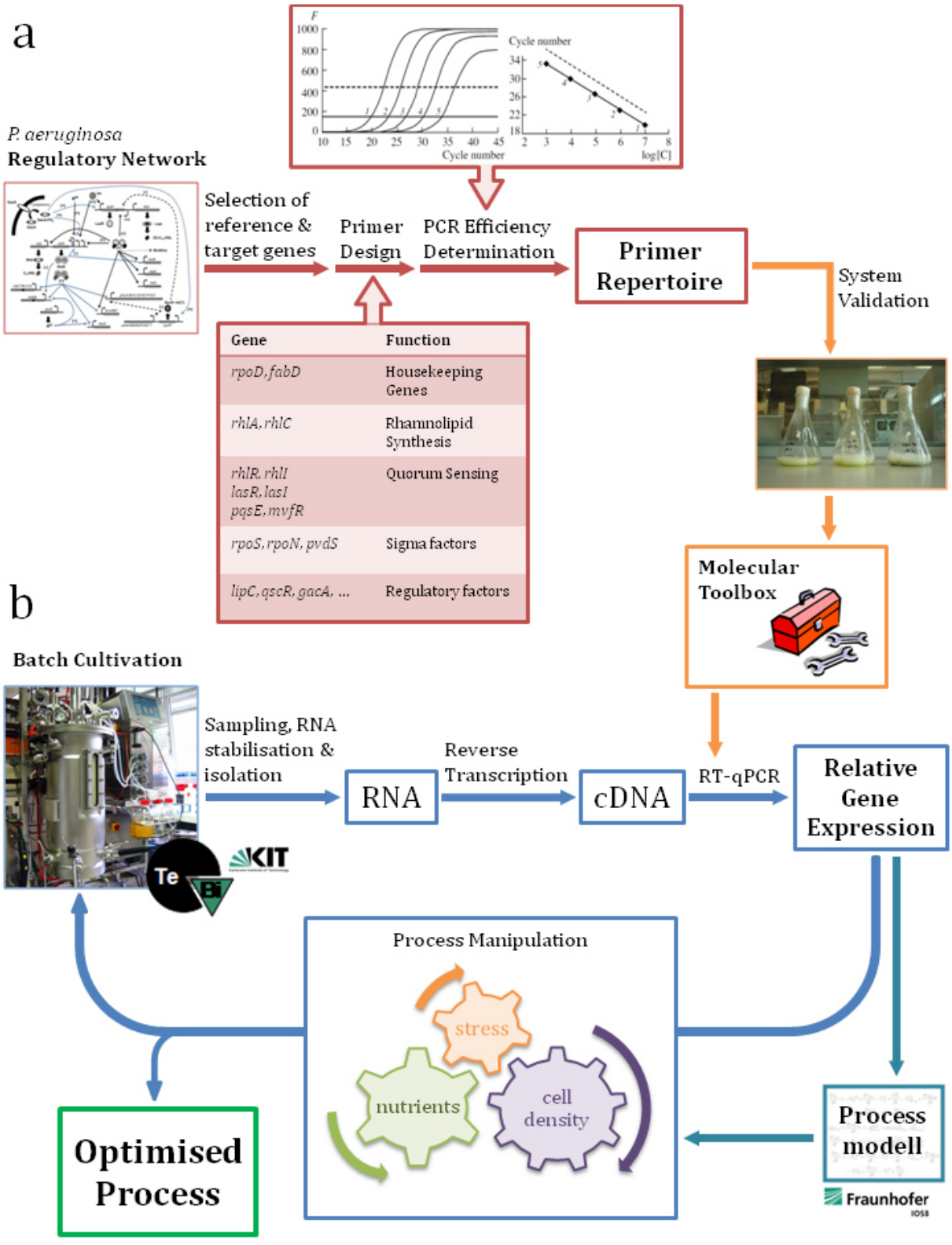

Fig. 1 Workflow for the molecular biology based optimisation of rhamnolipid production by *P. aeruginosa* PAO1 with method establishment (a) and application thereof, i.e. process optimisation (b)

In parallel, these gene expression data will be fed into a process model that was established by the Fraunhofer Institute of Optronics, System Technologies and Image Exploitation (IOSB) to describe the bioprocess, which will ultimately contribute to process optimisation. Several rounds of repeated evaluation of gene expression and process manipulation may eventually lead to an optimised process with improved rhamnolipid production.

On the one hand, expression profiles under standard conditions that is under nitrogen limitation with 125 g/L sunflower oil as sole carbon source were assessed during a batch process in a large-scale 42 L-bioreactor. The standard batch process used in this study has already been established and validated (Müller et al. 2010) and the production capacity for *Pseudomonas aeruginosa* PAO1 under these conditions was evaluated (Müller et al. 2011).

On the other hand, different cultivation scenarios were examined in shake flask experiments to find new ways to manipulating the bioprocess and enhance rhamnolipid synthesis. A first up-scale experiment with the most promising alteration, i.e. iron limiting conditions, was set up in a 2.2 L-bioreactor and gene expression profiles under altered conditions was assessed (Fig. 2).

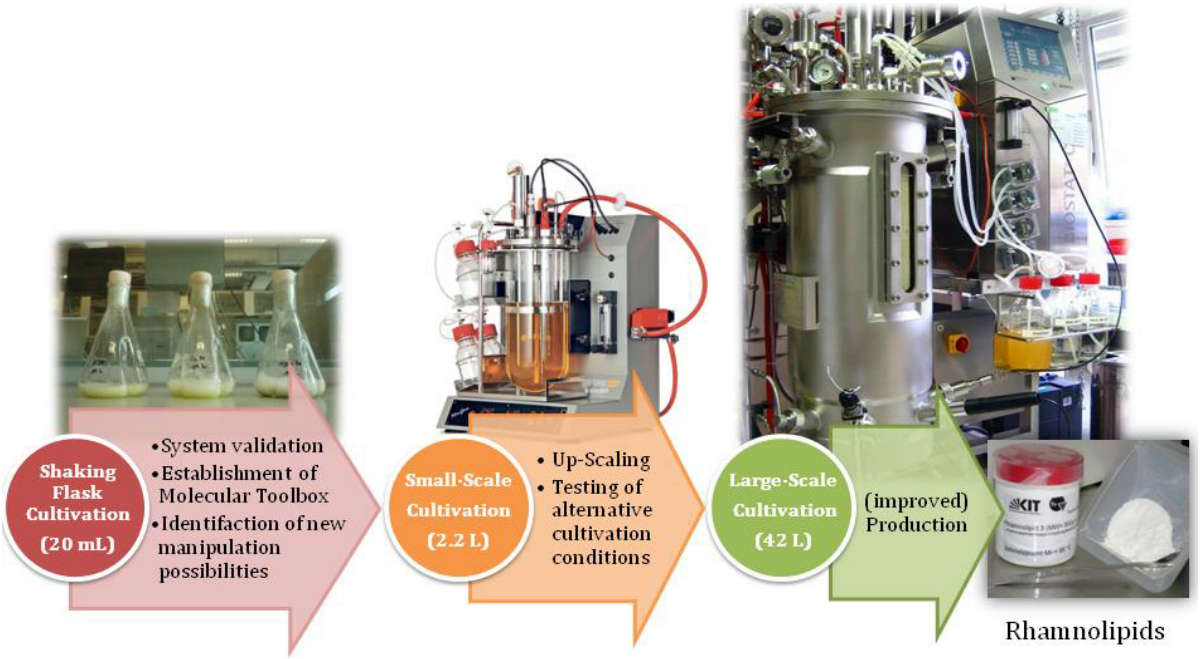

Fig. 2 Up-Scaling approach for rhamnolipid production by *P. aeruginosa* PAO1

iii. Introduction

Rhamnolipids are surface-active glycolipids that are produced by several *Pseudomonas* species, as well as bacteria from other families, classes or phyla (Abdel-Mawgoud et al. 2010). However, rhamnolipid production remains a characteristic for *Pseudomonas aeruginosa* (Rahman et al. 2010). Amongst the *P. aeruginosa* species, PAO1 is the best studied producer yet with a fully sequenced genome accessible online under www.pseudomonas.com (Stover et al. 2000; Winsor et al. 2009). Yet, reasons and mechanics behind important functions of rhamnolipids remain unclear. It has been speculated that rhamnolipid secretion was developed in the context of substrate uptake improvement, especially that of hydrophobic nature (Chrzanowski et al. 2012). Rhamnolipids connect the hydrophilic microorganisms with water-insoluble hydrocarbons thus causing an emulsification of the hydrophobic substrate and a hydrophobisation of the cell wall. Additionally, rhamnolipids alter the microbial cell surface resulting to an increased contact area between cells and substrate (Zhang and Miller 1995). Besides their antibacterial, antifungal and antiviral properties, rhamnolipids have versatile biotechnological applications ranging from bioremediation of contaminated soil and polluted waters over enhanced oil recovery to use in the cosmetic, pharmaceutical and detergent industries (Kim et al. 2012; Nguyen et al. 2008; Wang et al. 2007). This is mainly due to their excellent biodegradability, low aquatic toxicity, comparable physicochemical properties to synthetic surfactants and the ability to produce rhamnolipids from renewable or waste substrates (Abdel-Mawgoud et al. 2010; Marsudi et al. 2008; Rahman et al. 2002b; Syldatk et al. 1985). Therefore, rhamnolipids have gained more and more attention as they represent an interesting eco-friendly alternative to traditional, environmentally unfriendly, ecotoxic petrochemical surface-active components (Banat et al. 2000). While rhamnolipids were discovered more than 60 years ago by Jarvis and Johnson, industrial production has not yet been achieved in large scale, which is mainly due to low product yields, excess foaming, costly raw materials and expensive downstream processing (Banat et al. 2000; Jarvis and Johnson 1949). Additionally, the complex regulatory network behind rhamnolipid synthesis in *P. aeruginosa* constitutes a major obstacle in the way to upscale production

especially when it comes to expression in heterologous non-producing hosts. Attempts with recombinant *Escherichia coli* (Cabrera-Valladares et al. 2006) or *Pseudomonas putida* (Toribio et al. 2010) resulted in low product yields that were far below those achieved by its natural producer. However, Wittgens et al quite recently demonstrated good rhamnolipid production using the genetically optimised non-pathogenic safety-strain *Pseudomonas putida* KT2440 grown on glucose as carbon source (Wittgens et al. 2011). The highest yields so far have been reported by Giani, who claims to have developed a process that uses *Pseudomonas aeruginosa* strains DSM 7107 and DSM 7108 to produce yields between 70 and 120 g/L in their patent from 1997 (US5501966). However, no such yield was reported on laboratory scale ever since, which makes it even more crucial to fully understand the regulatory mechanisms behind rhamnolipid synthesis in *Pseudomonas aeruginosa*.

iv. *Pseudomonas aeruginosa* and Quorum Sensing

P. aeruginosa was initially discovered in the late 19th century (Fordos et al. 1859) and is a Gram-negative human pathogen, which is highly versatile (Stover et al. 2000) and able to adapt to numerous various habitats such as humans, water and soil (Banin et al. 2005). The large size of its genome with 6.3 million base pairs (Mbp) harbouring more than 5500 predicted open reading frames (ORFs) is due to the high genetic complexity, which nears that of simple eukaryotes and reflects the 'all-round nature' of *P. aeruginosa* (Silby et al. 2011; Stover et al. 2000). Together with *Burkholderia cepacia*, it is the major pathogen in Cystic Fibrosis (CF) (Eberl and Tummler 2004). Furthermore, *P. aeruginosa* is also known to cause a variety of both acute and chronic human infections in patients suffering from AIDS, lung cancer, pulmonary disease or CF (Balasubramanian et al. 2013).

Essential for chronic infections such as CF is quorum sensing (QS) - a bacterial system for the detection of population density and cell-to-cell communication (Waters and Bassler 2005). *P. aeruginosa* employs three such systems, i.e. the *las*, *rhl*, and *pqs* QS

systems (Juhas et al. 2005) (Fig. 3). Of these three systems, two are *N*-acyl homoserine lactone (AHL)-dependent, namely the *las* and *rhl* system, composed of the synthases LasI and RhlI and the regulators LasR and RhlR (Juhas et al. 2005; Schuster and Greenberg 2006). The corresponding homoserine lactones for the *las* and *rhl* systems are 3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl homoserine lactone (C4-HSL) respectively (Rahman et al. 2010) (Fig. 4). The mechanism of action is similar for all QS systems. The autoinducer is generated by an autoinducer synthase and binds to its cognate transcriptional regulator. Consequently, the active complex of autoinducer and regulator controls the transcription of its target genes (Waters and Bassler 2005). The significance of QS is made clear by the fact that approximately 10 % of the *P. aeruginosa* genome is regulated by QS (Venturi et al. 2006).

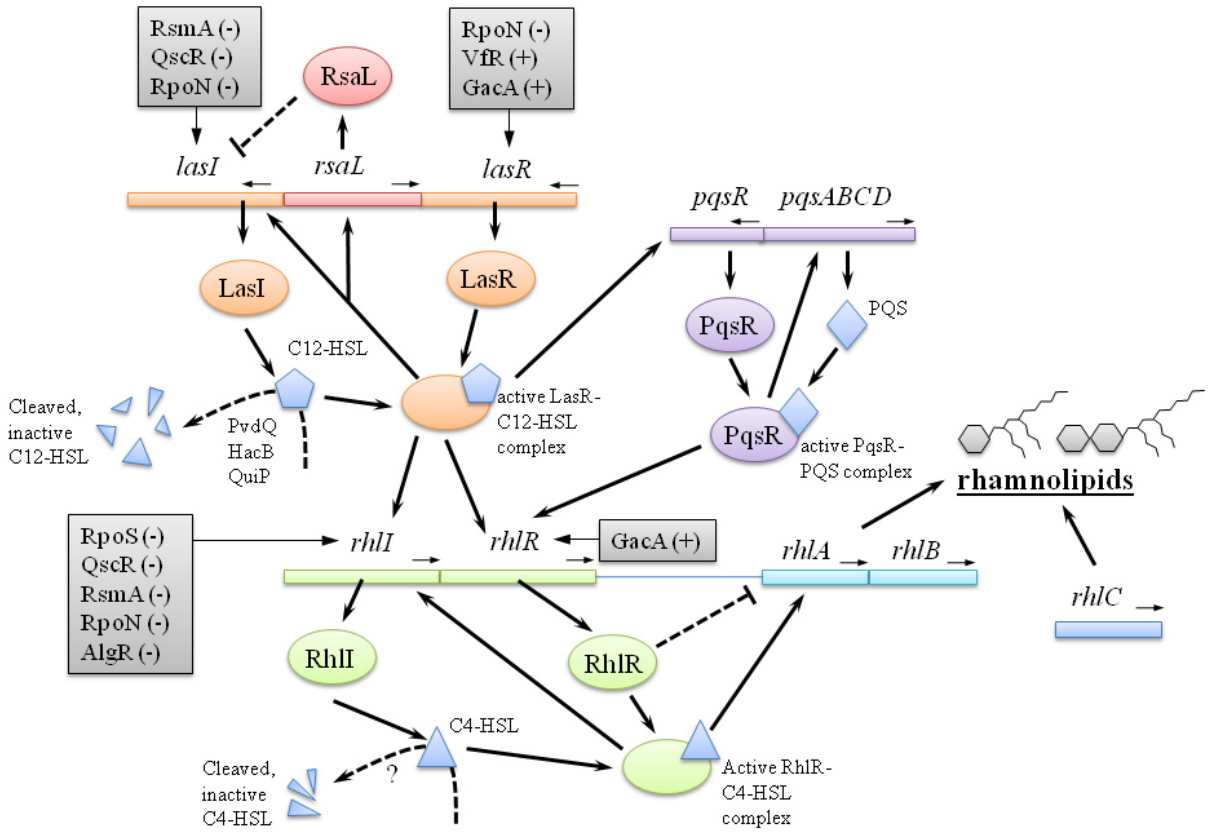

Fig. 3 AHL- and AQ-dependent Quorum Sensing networks in *P. aeruginosa*

A third, AHL-independent QS system based on 2-heptyl-3-hydroxy-4(1H)-quinolone, also referred to as *Pseudomonas* quinolone signal (PQS), has been described in *Pseudomonas* species (Pesci et al. 1999). PQS is recognised by the LysR-type transcriptional regulator PqsR (MvfR), which can also be activated by the PQS precursor 2-heptyl-4-quinolone (HHQ) although with 100-fold less affinity (Farrow et al. 2008). The structural genes for the *pqs* system components are organised in the *pqsABCDE* operon, which is required for the synthesis of various 2-alkyl-4-quinolone (AQ) signal molecules and is under the control of PqsR (Diggle et al. 2003; Williams and Camara 2009). The response effecter of the *pqs* system, PqsE, is not required for PQS biosynthesis and was shown to control the production of various virulence factors including rhamnolipids amongst others (Nadal Jimenez et al. 2012).

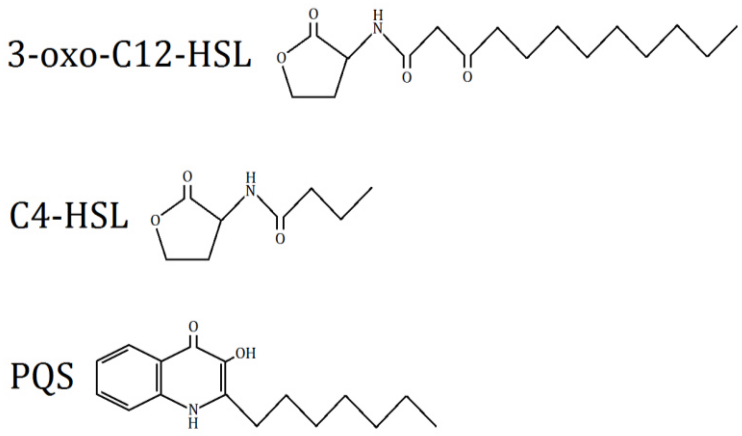

Fig. 4 Structures of *N*-acyl-homoserine lactone species 3-oxo-C12-HSL and C4-HSL, and the 2-alkyl-4-quinolone PQS produced by *P. aeruginosa* as part of quorum sensing

The three QS systems are intimately linked to each other in a hierarchically fashion (Nadal Jimenez et al. 2012). At this, the *las* system directly controls the *rhl* system, as the activated 3-oxo-C12-HSL-LasR-complex was shown to directly up-regulate *rhlR* transcription (Latifi et al. 1996). The PQS system again acts as a link between the *las* and *rhl* systems that activates the *rhl* system in a 3-oxo-C12-HSL-LasR independent manner (McKnight et al. 2000). Furthermore, both the *las* and *rhl* systems act as positive

feedback loops upon themselves, since 3-oxo-C12-HSL-LasR and C4-HSL-RhlR positively regulate *lasI* and *rhlI* expression respectively and hence production of their cognate autoinducer (Müller and Hausmann 2011; Williams and Camara 2009). Further layers of regulation are provided by a negative feedback loop through the transcriptional repressor RsaL, which is up-regulated by activated LasR and directly represses *lasI* and *rsaL* transcription (Rampioni et al. 2006; Rampioni et al. 2007; Rampioni et al. 2009). Furthermore, long chain AHL acylases, i.e. PvdQ and QuiP, add yet another layer of QS homeostasis by degradation of 3-oxo-C12-HSL (Huang et al. 2006; Sio et al. 2006).

v. Rhamnolipid Biosynthesis Pathway in *P. aeruginosa*

Rhamnolipids are glycolipids that are composed of a polar head group that contains one or two units *L*-rhamnose and one or more hydrophobic, non-polar tails that consist of lipid chains with varying chain lengths (Rahman et al. 2010). Depending on the number of rhamnose units, rhamnolipids are specified as mono- or dirhamnolipids, respectively (Müller et al. 2011). While some species produce solely mono-rhamnolipids, others only produce dirhamnolipids, yet others produce a mixture of both. *Pseudomonas aeruginosa* is known to produce four types of rhamnolipid species, namely RL1 ($RhaC_{10}C_{10}$), RL2 ($RhaC_{10}$), RL3 ($Rha_2C_{10}C_{10}$) and RL4 (Rha_2C_{10}) (Rahman et al. 2002a). The most frequently mono- and dirhamnolipids produced by *P. aeruginosa* are α-*L*-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate (RL1) and α-*L*-rhamnopyranosyl-(1-2)-α-*L*-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate (RL3) (Müller et al. 2011) (Fig. 5a).

The single components for rhamnolipids, namely rhamnose units and fatty acid chains are synthesised in separate biochemical pathways, which ultimately converge to form rhamnolipids. While the precursor for the polar head group, dTDP-*L*-rhamnose is generated via gluconeogenesis, the lipid chains are synthesised *de novo* and result in a precursor molecule called β-hydroxyalkanoyl-β-hydroxyalkanoyl (HAA) S-CoA (Airoldi et al. 2007). This precursor is specific for each rhamnolipid specie and catalysed by

rhamnosyltransferase 1 chain A (RhlA) (Deziel et al. 2003) (Fig. 5b). Transfer of dTDP-*L*-rhamnose onto HAA and hence synthesis of mono-rhamnolipid is then catalysed by the membrane-bound rhamnosyltransferase 1 chain B (RhlB) (Soberon-Chavez et al. 2005). Dirhamnolipids are subsequently generated from mono-rhamnolipids by transfer of an additional dTDP-*L*-rhamnose onto an existing mono-rhamnolipid molecule, which is catalysed by the rhamnosyltransferase 2 (RhlC) (Rahim et al. 2001; Soberon-Chavez et al. 2005) (Fig. 5b).

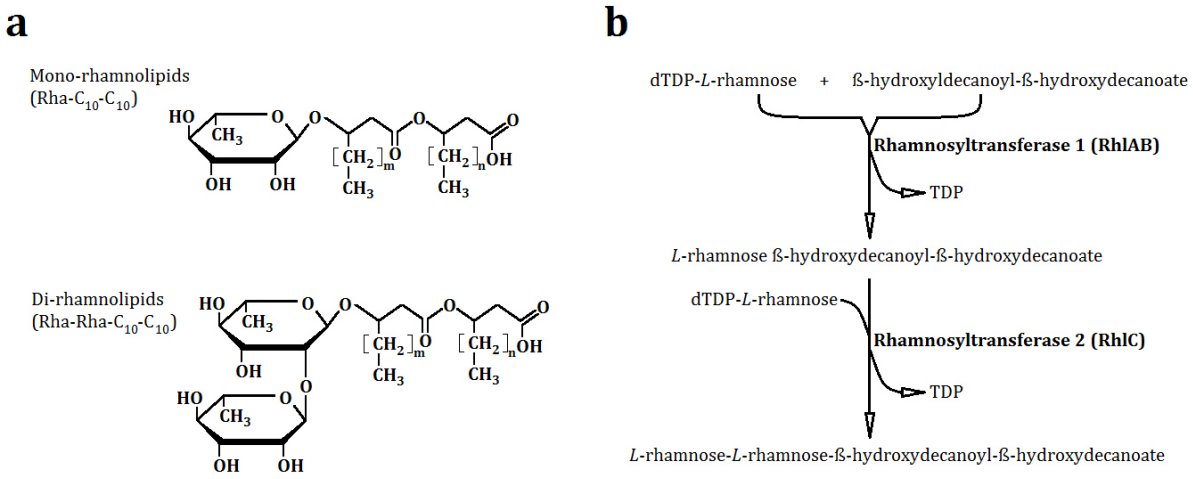

Fig. 5 General structure of mono- and dirhamnolipids (a) and biosynthesis pathway (b) in *P. aeruginosa*. m,n = 4-8 (adapted from Müller 2011 and Rahim 2001)

vi. Regulation of Rhamnolipid Synthesis in *P. aeruginosa*

The genes for the two rhamnosyltransferase 1 chains, namely *rhlA* and *rhlB,* are encoded on one single operon, the *rhlAB*-regulon, and are hence co-expressed (Sullivan et al. 1998). Both the *rhlAB*-operon and the *rhlC* gene are under the control of growth-dependent QS, and rhamnolipid production has been shown to be directly linked to the *rhl* QS system via the regulator RhlR (Medina et al. 2003c). However, several other factors beside cell density seem to influence *rhlAB* as well as *rhlC* expression and it has

become evident that the entire QS system itself and hence rhamnolipid synthesis are subjected to additional layers of regulation, as most QS-controlled genes show a pattern of delayed expression and so does *rhlAB* (Schuster and Greenberg 2006). The *rhlAB* operon is also partially dependent on the alternative stationary phase sigma factor RpoS (σ^{38} or σ^{S}) (Medina et al. 2003b). Other studies link rhamnolipid synthesis to nutritional conditions, such as nitrogen exhaustion and the alternative sigma factor for nitrogen limitation, RpoN (σ^{54}), has been shown to be involved in the regulation of *rhlAB*, as well as *rhlR* and *rhlC* expression (Medina et al. 2003a; Medina et al. 2003c; Rahim et al. 2001). However, the *rhlAB* promoter itself has been demonstrated to be independent of RpoN. The dependency of *rhlAB* expression on this sigma factor might occur indirectly through RhlR, whose gene harbours a RpoN-dependent transcriptional start site (Medina et al. 2003c). This allows co-ordinate expression and hence production of dirhamnolipid only when its precursors, mono-rhamnolipids, are present (Rahim et al. 2001). Several other QS-related and unrelated transcriptional, as well as posttranscriptional regulation mechanisms that influence rhamnolipid synthesis have been described and are reviewed by Müller (2011).

vii. Rhamnolipid Production and Iron in *P. aeruginosa*

Iron constitutes an essential trace element for many species and iron availability is of major importance not only for *P. aeruginosa,* but for many other bacteria as well (Cornelis et al. 2010). The competition for iron between pathogens and its host is often critical for the outcome of a disease (Skaar et al. 2010). For *P. aeruginosa*, iron is furthermore one of the major turnstiles when it comes to physiology and its availability is responsible for crucial changes in lifestyle and growth behaviour (Fig. 6).

Under aerobic conditions, which Pseudomonads as aerobic microorganisms are most likely to encounter naturally, iron occurs in its oxidised form, Fe^{3+}, ferric iron (Vasil and Ochsner 1999). Unlike the soluble ferrous iron Fe^{2+}, Fe^{3+} is poorly soluble under these aerobic conditions (Braun and Killmann 1999). Furthermore, iron uptake requires tight regulation, since both forms are highly reactive in its free form and are able to form

potentially harmful reactive oxygen species (Andrews et al. 2003). In turn, many genes of *P. aeruginosa* including those for iron uptake or several virulence factors are subjected to iron-dependent regulation (Frangipani et al. 2013).

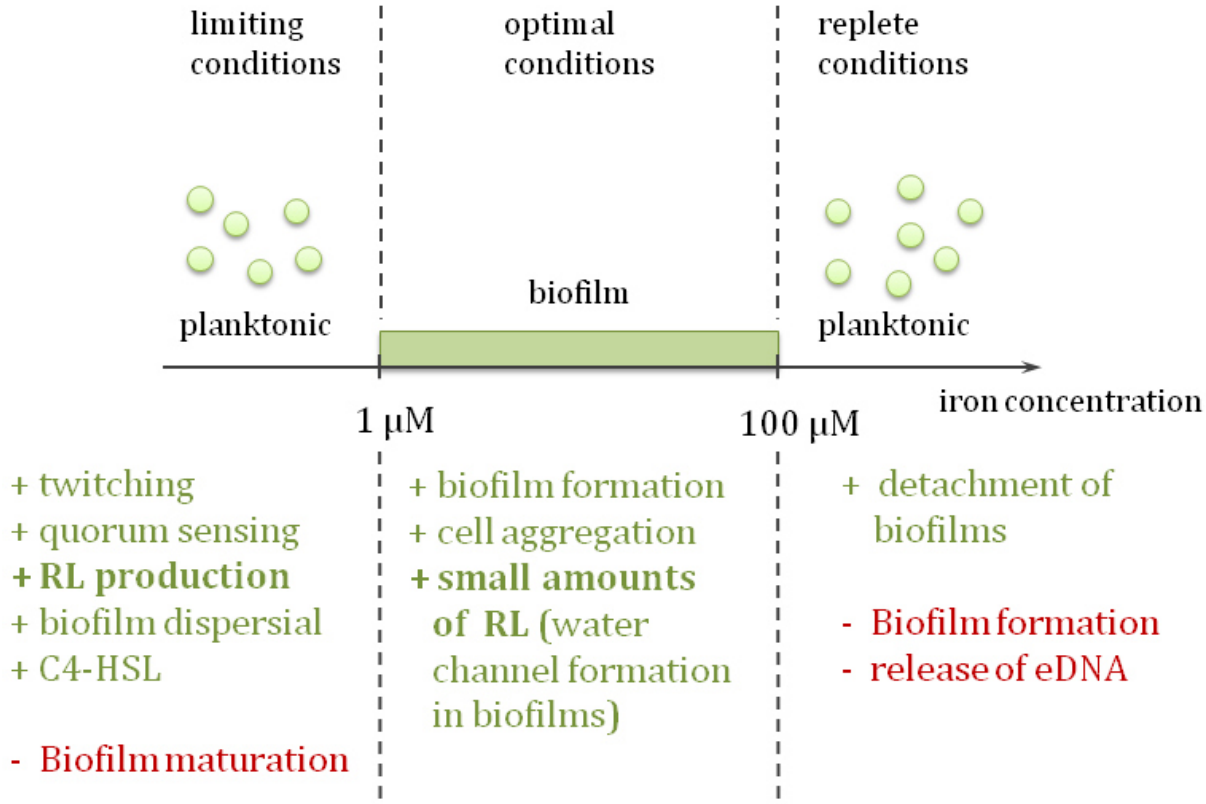

Fig. 6 Influence of iron on the lifestyle of *Pseudomonas aeruginosa*. Under iron limiting conditions below 1 µM, the planctonic lifestyle is favoured, whereas between 1 and 100 µM, the biofilm mode of growth is favoured. Above 100 µM on the other hand, the planctonic lifestyle is favoured again. Common laboratory use for iron replete conditions varies between 10 and 100 µM (Berlutti et al. 2005; Glick et al. 2010; Musk et al. 2005; Patriquin et al. 2008; Vasil and Ochsner 1999; Yang et al. 2007)

V. Materials and Methods

Bacterial Strains

Pseudomonas aeruginosa PAO1 (DSM 22644) was used for batch cultivations, as well as the planktonic cultures.

Media

Small scale planktonic cultures were grown in standard BM2 medium at 37 °C in a rotary shaker (MaxQ 4000, Thermo Scientific) at 170 rpm. BM2 medium was composed of a 10x phosphate ammonia base containing 0.07 M $(NH_4)_2SO_4$, 0.4 M K_2HPO_4 and 0.22 M KH_2PO_4, as well as 40 % glucose, 200 mM $MgSO_4$ x 7 H_2O and 10 mM $FeSO_4$ x 7 H_2O. All components were sterilised by autoclaving, except for $FeSO_4$, which was filter sterilised using a 0.2-µm membrane filter (VWR, Karlsruhe, Germany).

For the large-scale batch cultivation under standard conditions using a 42 L-bioreactor, *P. aeruginosa* PAO1 was grown in a nitrogen limited production medium with 125 g/L sunflower oil as sole carbon source. The medium was furthermore supplemented with a Ca^{2+}-free mineral salt solution containing 15 g/L $NaNO_3$, 0.5 g/L $MgSO_4$ x 7 H_2O, 1 g/L KCl and a 0.1 M sodium phosphate buffer. Cultivations were conducted at pH 6.5, whereas 4M NaOH or 4M H_3PO_4, respectively, were used to adjust pH levels. Additionally, 1 mL/L trace element solution was added in total. The trace element solution was composed of 2.0 g/L sodium citrate x 2 H_2O, 0.28 g/L $FeCl_3$ x 6 H_2O, 1.4 g/L $ZnSO_4$ x 7 H_2O, 1.2 g/L $CoCl_2$ x 6 H_2O, 1.2 g/L $CuSO_4$ x 5 H_2O and 0.8 g/L $MnSO_4$ x H_2O. After preparation, the solution was filter sterilised with a 0.22-µm membrane filter (Carl Roth GmbH, Karlsruhe, Germany). The batch cultivation was according to Müller (2011).

Preparation of Seed Cultures for large-scale Batch Cultivation

Pre-cultures of *P. aeruginosa* PAO1 were prepared from glycerol stocks using a total volume of 100 µL stock solution in 25 mL Luria Bertani (LB). Over-night cultivation was conducted at 37 °C in 250 mL baffled shake flasks at 120 rpm (Multitron II, HT Infors, Bottmingen, Switzerland). Prior to batch cultivation, 5 mL of the pre-culture were inoculated in 200 mL production medium supplemented with 25 g ($\delta_{\text{sunflower oil}}$ = 0.921 g/ mL) sunflower oil at 37 °C in 1 L baffled shake flasks at 120 rpm. After 24 h of incubation, an appropriate volume of this culture was used to inoculate the bioreactor. For this purpose a theoretical OD_{580} of 0.06 was used.

Production of Rhamnolipids using *P. aeruginosa* PAO1 in 42-L Bioreactor scale

For large-scale batch cultivation, a 42-litre stirred tank bioreactor, with a nominal working capacity of 30 litres was used (Biostat® Cplus, Sartorius Stedim Biotech, Göttingen, Germany). The bioreactor featured an integrated process control system for pH, temperature, pO_2, airflow, and foam level regulation (MFCS/win 2.1, Sartorius BBI, Göttingen, Germany). Cultivation was conducted at 37 °C under a constant pO_2 of 5 % at a stirring rate of 400 rpm. The bioreactor was equipped with a Rushton-turbine for dispersion of air bubbles and mixing, propeller-type stirrers for axial mixing, and a specifically designed foam stirrer to ensure homogeneity of the suspension. Minimal dissolved oxygen (pO_2) was controlled by varying the aeration rate between 0.1 and 3.3 L/ (L x min). To control excess foaming, a mechanical foam breaker was installed in the headspace of the bioreactor. Trace element solution was added four times over the whole cultivation process, namely at the start of cultivation, as well as 20, 40 and 70 h during cultivation. Furthermore, pH level was controlled and if necessary adjusted to pH 6.5 using 4M NaOH or 4M H_3PO_4 respectively. The batch cultivation was according to Müller (2011).

Iron-limited Batch Cultivation of *P. aeruginosa* PAO1 in a 2.2-L Bioreactor scale

Batch cultivations of *P. aeruginosa* PAO1 DSM22644 were carried out in a benchtop bioreactor (MiniFors, Infors HT, Bottmingen, Switzerland) equipped with a 2.2 L glass vessel and a Rushton-turbine as a stirring device. Cultivations were performed with a total liquid volume of 800 mL in production medium and sunflower oil as a sole source of carbon, as described previously (Müller et al. 2010), omitting iron in the initial addition of trace elements and all subsequent supplementations. To account for foaming during cultivation, a 5 L stirred foam trap containing 4 M H_3PO_4 was connected to the exhaust gas cooler. To minimise iron contamination of the media, shrink tubing was applied to cover metallic surfaces of the stirring shaft, baffles and aeration tube. pH was maintained at 6.50 with 4 M NaOH and 4 M H_3PO_4, and the pO_2 was controlled by varying stirrer speed from 500 rpm to 1200 rpm at a constant aeration rate of 0.2 L/ (L x min).

Shake Flask Cultures under Iron Limitation

Iron starvation was achieved by omitting Fe^{3+} in the BM2 medium, while iron repletion was achieved by using the tenfold amount of Fe^{3+} compared to standard BM2, where iron is 10 µM. Iron titration was done by varying the amount of Fe^{3+} in the medium ranging from 1, 2, 5, 10, 20 to 100 µM. Nitrogen limiting conditions were achieved by using one half or one tenth of the ammonium sulphate concentration that is used in standard BM2.

Sampling

Samples for offline molecular analysis of the batch cultivations were taken approximately every five to six hours, starting after sufficient biomass accumulation. Gene expression ratios were then calculated relatively to this first time point of sampling. RNA stabilisation was conducted on-site using RNAProtect® Bacteria Reagent from Qiagen (Hilden, Germany) according to manufacturer's instructions. Samples were

stored at -80 °C until sample preparation. Furthermore, samples for offline analysis of bio dry mass (BDM), sunflower oil, mono-/ dirhamnolipid and nitrate concentration were routinely taken as described by Müller, 2011. Briefly, culture broth was mixed with equal volumes of n-hexane to remove excess sunflower oil. Subsequently, mass of sunflower oil was obtained gravimetrically from the organic phase. Rhamnolipid quantification was done as described previously (Müller et al. 2011). Biomass concentration was obtained gravimetrically from 3 samples, with the highest relative error of 13.8 %. The concentration of rhamnolipid was determined by HPLC analysis, with a maximum error of 7.1 %.

RNA Isolation and Reverse Transcription

Total RNA was isolated after RNA stabilisation by phenol/chloroform extraction using Qiagen's QIAzol® Lysis Reagent in combination with the RNeasy® Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was subsequently digested using TURBO® DNase (Invitrogen, Darmstadt, Germany) and successful digestion was then evaluated by using polymerase chain reaction followed by loading eventual products on an agarose gel. To control RNA integrity as well as quality, 260/280 and 260/230 ratios were assessed photo-spectrometrically (NanoDrop® ND-1000, Thermo Scientific, Frankfurt, Germany) and isolated RNA was additionally loaded on agarose gels. RNA concentrations were then measured and a total of 250 ng RNA was used for cDNA synthesis. For each sample, an equal amount of RNA was transcribed into cDNA. Random-primed reverse transcription was conducted using MultiScribe® Taqman Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. The temperature profile was according to protocol (25 °C, 10 minutes; 48 °C, 30 minutes; 95 °C, 5 minutes; 4 °C, ∞). After reverse transcription, samples were directly used for real-time PCR or otherwise stored at -20 °C.

PCR Efficiency Calculation using Quantitative Polymerase Chain Reaction (qPCR)

Serial dilutions (1:10) of target DNA were prepared from 50 to 10 ng/µL solutions of cDNA. C_t values for PCR efficiency calculation were obtained using the ABI PRISM® 7300 and the associated SDS Software (v1.4, Applied Biosystems, Darmstadt, Germany). Subsequently, C_t values were analysed using the Qbase Plus software from BioGazelle (Zwijnaarde, Belgium). The primer pairs were analysed using the standard curve method by plotting the C_t values against N_0 and subsequently calculating the efficiency using the following equation:

$$E = 10^{\frac{-1}{slope}} - 1$$

Gene Expression Analyses using qPCR

Gene expression was assessed using a SYBR Green mediated two-step quantitative real-time PCR. Prior to PCR, RNA was first transcribed into cDNA as described above and in a second step, the quantitative real-time PCR was performed using an ABI PRISM® 7300 (Applied Biosystems, Darmstadt, Germany) in combination with KAPA® SYBR FAST qPCR ABI PRISM (PEQLAB, Erlangen, Germany). Primers used for real-time PCR are listed in Table 1. Prior to gene expression analyses, primers were checked for appropriate product amplification and primer efficiencies were determined. All primers used met the requirements as well as the tolerable efficiency range of 90 to 110 %. Gene expression data was obtained using the ABI PRISM® 7300 associated SDS Software (v1.4, Applied Biosystems, Darmstadt, Germany) and subsequently analysed using the Qbase Plus software from BioGazelle (Zwijnaarde, Belgium), which calculates relative gene expression based on the delta-delta Ct method (Pfaffl 2001; Hellemans et al. 2007). As reference genes for relative quantification calculations served the housekeeping genes, *rpoD*, the major sigma (σ^{70}), and *fabD*, the malonyl-CoA-[acyl-carrier-protein]-transacylase, which previously have been shown to be stably expressed in *P. aeruginosa* and are hence considered suitable as reference genes (Savli et al. 2003; Potvin et al. 2008).

Regarding the expression profiles for the batch cultivations, equal amounts of cDNA were used for each sample. The expression of a particular target gene at a certain time point during the cultivation was compared with the expression of the same target gene at the start of the process that is after 16 hours of cultivation. Therefore, the expression of all target genes of interest at sixteen hours is considered to be one. This expression was then normalised against the two reference genes. For shake flasks experiments, gene expression of target genes in standard BM2 medium served as a reference.

Data Analysis

For the analysis of biomass growth, a fourth-degree polynomial equation was used to fit the data. For the analysis of rhamnolipid production, a logistic equation with four parameters was used to fit the data. The formula used for the logistic growth is described by Müller, 2010. Calculations were done using a scientific data analysis and graphing software (SigmaPlot 9.0, Systat, San José, USA). Specific (μ) and maximum (μ_{max}) growth rates were calculated based on the derivation of the polynomial fitting. Analogue to this, the specific production rate of total rhamnolipids per BDM ($q_{RL, spec}$) was calculated based on the BDM and rhamnolipid fittings. Concentrations of total rhamnolipids were obtained by adding concentrations of mono- and dirhamnolipids.

***RhlA_gfp* and *rhlC_gfp* expression analyses**

The *rhlA_gfp* fusion vector has been previously described (Lequette et al. 2005). Briefly, the *rhlA* promoter region was amplified from chromosomal *P. aeruginosa* PAO1. The amplified product extends 551 bp upstream of the *rhlA* translational start and was cloned in front of the promoterless gfp in pPROBE-AT vector. This *rhlA_gfp* fusion vector, transformed into *Escherichia coli* DH5α was kindly provided by Professor Greenberg from the University of Washington, USA. Subsequently, the *rhlA_gfp* fusion vector was isolated using the GeneJet PCR Purification Kit (Fermentas, Thermo Scientific, Frankfurt, Germany), checked for presence of insert and vector by digesting it with EcoRI and HindIII and introduced into competent *P. aeruginosa* PAO1 H103 wild type strain for gfp

measurement experiments. The *rhlC_gfp* fusion strain was constructed as follows. The *rhlC* promoter region was amplified from *P. aeruginosa* PAO1 H103 chromosomal DNA by PCR with 5'-aaaaaaagcttAGAGGCTGGTCGTGGACA-3' and 5'-aaaaaggtaccACGGCAGG TAGCTTTCCTTT-3' using the KAPA HiFi Fidelity polymerase from (Peqlab, Erlangen, Germany). The cloned fragment is 273 bp and extends from 254 bp upstream of the *rhlC* translational start containing the AGGAGG-Shine-Dalgarno sequence for ribosome binding. The amplified product was then isolated from an agarose gel using the GeneJET PCR Gel Extraction Kit (Fermentas, Thermo Scientific, Frankfurt, Germany) according to the manufacturer's instructions. The product was then double-digested with KpnI and HindIII (Fermentas, Thermo Scientific, Frankfurt, Germany) using Kpn buffer. The gfp containing vector, pPROBE-AT', was digested with KpnI and HindIII separately from the insert under same reaction conditions. Successful digestion was controlled by an agarose gel after purifying the fragments using the GeneJet PCR Purification Kit (Fermentas, Thermo Scientific, Frankfurt, Germany). Vector and insert were subsequently ligated using T4 DNA Ligase (Fermentas, Thermo Scientific, Frankfurt, Germany) to generate the *rhlC_gfp* fusion vector. Subsequently, transformation of the *rhlC_gfp* fusion vector into competent *E. coli* DH5α was done by heat transformation. Positive selection was conducted on LB agar containing ampicillin (100 mg/mL). The *rhlC_gfp* fusion vector was consequently isolated using the GeneJET Plasmid Miniprep KIT (Fermentas, Thermo Scientific, Frankfurt, Germany) and introduced into competent *P. aeruginosa* PAO1 H103 wild type strain using electroporation. Positive clones were subsequently selected using LB agar containing carbenicillin (300 mg/mL). For the gfp, as well as the growth measurements, an Infinite F200 Pro microplate reader and associated software (Tecan Group Ltd.) were used. The emission wavelengths were 485 and 535 nm for gfp fluorescence and 600 nm for absorbance measurements, respectively. The measured gfp fluorescence emission was then correlated to the measured optical density of each culture to produce a specific gfp fluorescence.

Rhamnolipid quantification using Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS)

Mono- and dirhamnolipids were quantified in the cell-free culture supernatant of the shake flask experiments using liquid chromatography tandem mass spectrometry (LC/MS/MS). For this purpose, culture supernatants were treated with chloroform to extract rhamnolipids (3:5), which are consequently located in the organic phase. After freezing the samples and subsequent phase separation, the organic phase was isolated and chloroform evaporated using a heating block and a stream of gaseous N_2. The remaining dry substance was then resuspended in CH_3CN/ 10 mM NH_4OAc (50:50) and samples analysed using an API 365 LC/MS/MS System (Perkin Elmer). Purified mono- and dirhamnolipid served as a reference for calibration. The rhamnolipid quantification was conducted by Michael Nusser (Karlsruhe Institute of Technology, Institute for Functional Interfaces).

Preparation of Iron Chelators

Stock solutions of iron chelators were 10 mM for acetohydroxymic acid, picolinic acid, protocatechuic acid, and EDTA, 30 mM for 2,2'-bipyridine and 10 mg/mL for lactoferrin and were prepared by weighing in several milligrams of a given chelator. Acetohydroxamic acid was obtained from Alfa Aesar, Karlsruhe, Germany and protocatechuic acid was obtained from HWI Analytic GmbH. Picolinic acid, FerroZine™, lactoferrin from bovine milk, EDTA, and 2,2’-bipyridine were obtained from Sigma-Aldrich Chemie GmbH. All stock solutions were filter sterilised using a 0.2-µm membrane filter (VWR, Karlsruhe, Germany). BM2 medium was prepared as described above and chelators were administered in a ratio of 50:1 prior to incubation with bacteria so that all ferric iron would be fully chelated before cultivation.

VI. Results and Discussion

i. Establishment of Molecular Tools for Gene Expression Analyses using Quantitative Real-Time PCR

The underlying method for gene expression analyses in this thesis is quantitative Polymerase Chain Reaction (qPCR), a frequently used approach to analyse regulatory networks. For that, different aspects have to be taken into account, when the method is used for gene activity measurements.

PCR efficiency is on the one hand dependent on individual parameters, such as reaction components (i.e. $MgCl_2$, DNA polymerase, template), temperature, and time profiles (Nogva and Rudi 2004). On the other hand, efficiency of primer binding, i.e. the extent and stability of binding to the template, plays a crucial role, which makes a careful primer design an important prerequisite.

The efficiency of a PCR reaction can be expressed as

$$N_n = N_0 E^n \qquad \textit{Equation 1}$$

where *n* is the number of cycles; N_0 is the amount of initial reaction products; N_n is the amount of reaction products after *n* cycles, and E is the efficiency, whereas $1 < E < 2$.

Under optimum conditions, PCR efficiency nears two, as after each cycle the amount of starting material is doubled. However, this is not the case for all PCR reactions and the actual efficiency is not 100 % (Rebrikov and Trofimov 2006).

The PCR efficiency can also be expressed as:

$$N_n = N_0(1 + E^n) \qquad \textit{Equation 2}$$

where $0 < E < 1$ and E can be expressed as a percentage ($\%E = E \cdot 100\ \%$). In contemporary literature, both variants are applied (Pfaffl 2001; Peirson et al. 2003; Rutledge and Cote 2003; Wilhelm et al. 2003).

To determine PCR efficiency, a standard curve can be used based upon the amplification of known amounts of target DNA in serial dilutions (Rutledge et al. 2003). The amount of reaction products after n cycles, N_n, can be determined by introducing the threshold cycle C_t. The threshold cycle, also designated as C_t value, is the cycle, at which the reporter fluorescence is first detected reliantly. The equation for the standard curve can be derived mathematically by rearranging equation 2, where the number of cycles is substituted by the cycle threshold:

$$\log(N_0) = -\log(1 + E)\ x\ C_t + \log(N_t) \qquad \textit{Equation 3}$$

which has the general structure of a linear function y = mx + b, so that plotting $\log(N_0)$ versus C_t gives a line, from which the efficiency can easily be determined as:

$$slope = -\log(1 + E)$$

$$E = 10^{-slope} - 1 \qquad \textit{Equation 4}$$

where E is the slope-derived efficiency (equation 4).

Alternatively, the C_t value can be plotted against $\log(N_0)$ (Fig. 1). The equation for the slope-derived calculation of the efficiency thus becomes:

$$E = 10^{\frac{-1}{slope}} - 1 \qquad \textit{Equation 5}$$

where the increase of DNA amount during each cycle of replication is indicated by the *1/slope* value.

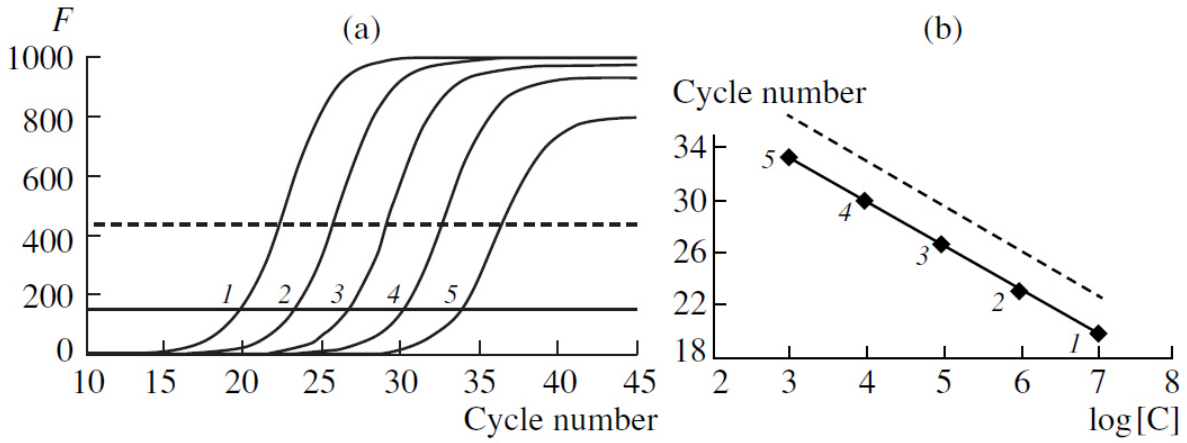

Fig. 1 Serial dilution method for determination of PCR efficiency. (a) Serial dilutions of DNA are analysed by plotting the measured fluorescence *F* of DNA accumulation versus the number of cycles. The fluorescence threshold is indicated by a solid black line, whereas the dashed black line is a variation thereof. (b) Resulting standard curves are based on data of the curves depicted on the left and are generated by plotting the cycle at the fluorescence threshold (=C_t value or cycle threshold) against the log of target DNA concentration. Adapted from Rebrikov et al. 2006

The serial dilutions and PCR efficiencies of the primer pairs for the two rhamnolipid synthesis genes, *rhlA* and *rhlC*, are shown exemplary in the following for the efficiency calculation of all primers used in this study. The mean C_t values obtained for the dilution series of the primers for *rhlA* and *rhlC* are shown in table 1. Serial dilutions (1:10) of target DNA were prepared from 10 ng/µL solutions of complementary DNA (cDNA) and included 5 log units. The RNA for the preparation of cDNA was derived from pure cultures of *P. aeruginosa* PAO1.

Table 1 Mean C_t values for PCR efficiency calculation for genes for rhamnolipid synthesis

log cDNA	*rhlA*	*rhlC*
-5	undet.	38.00
-4	undet.	39.00
-3	35.14	undet.
-2	31.89	32.60
-1	28.72	27.51
0	24.81	23.78

Consequently, those values were plotted against the log cDNA input and linear regression was applied to obtain the slope for the efficiency calculation (Fig 2a and b). For *rhlA*, a slope of -3.4172 and for *rhlC*, a slope of -3.34 was determined. An ideal slope of -3.33 yields an efficiency of 100 %. Following equation 5, the determined slopes resulted in an efficiency of 96.2 % and 99.3 % respectively. The determined PCR efficiencies for the remaining genes are given in table 2.

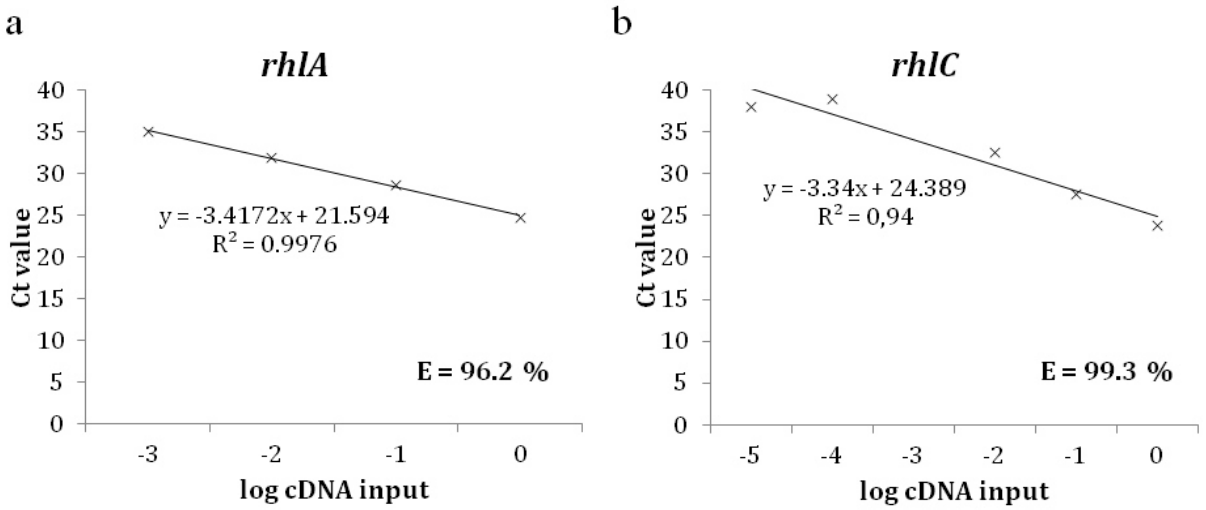

Fig. 2 PCR efficiency of the genes for the rhamnosyltransferases 1 and 2 (*rhlA* and *rhlC,* respectively) in *P. aeruginosa* PAO1. PCR efficiencies of *rhlA* (a) and *rhlC* (b) were determined using the serial dilution method and standard curves were generated by plotting the cycle at the fluorescence threshold (C_t value) against the log of target DNA concentration. The efficiency in percent was calculated from the slope of the regression curve and is indicated by *E* in the graph

The recommended range for primer efficiency for qPCR is 90 to 110 % (Real-Time PCR Handbook, Life Technologies, Darmstadt, Germany). An efficiency of more than 100 % can be due to many reasons, such as the presence of polymerase enzyme activators, unspecific products and primer dimers as well as improper dilution series, pipetting errors and contamination. Another reason might be due to artefacts in the DNA/RNA samples resultant of PCR inhibition caused by excess amount of DNA, ethanol, saccharides, phenols or other secondary metabolites. The presence of inhibitors results in a higher amount of cycle numbers that are needed to reach threshold cycle than in

samples without inhibitors. In diluted samples, inhibitors are also diluted and at some point do not have an inhibitory effect on the polymerase any longer. This results in smaller differences in C_t values between higher and lower diluted samples. Consequently, this leads to a steeper slope of the regression curve and hence a higher efficiency.

Table 2 PCR efficiencies of primers used for quantitative Real-Time PCR

Gene name	Efficiency [%]	Gene name	Efficiency [%]
algR	99.1	***qscR***	102.4
bqsS	109.7	***rhlA***	96.2
bqsR	91.6	***rhlC***	99.3
fabD	95.0	***rhlI***	106.0
fur	105.4	***rhlR***	105.6
gacA	89.4	***rpoD***	106.8
lasI	87.8	***rpoN***	106.7
lasR	110.2	***rpoS***	105.3
lipC	106.4	***rsaL***	99.8
mvfR	109.5	***rsmA***	93.1
pqsE	110.2	***vfR***	101.3
pvdS	117.5		

In general, the principle of relative quantification is very rational: the more copies of a target, the less number of cycles are needed to generate the number of amplicons that are necessary to reach the fluorescence threshold. However, an internal control has to be applied to account for the two major sources of variation in gene expression measurements. These are gene-specific (biological) variation on the one hand and non-specific (technical) variation including RNA extraction yield, RNA quantity and quality, reverse transcription efficiency and PCR efficiencies on the other (Bustin and Nolan 2004; Huggett et al. 2005). The most appropriate normalisation strategy is the use of reference genes (Vandesompele et al. 2002; Bustin et al. 2005). Reference genes for normalisation are usually housekeeping genes, which are stably expressed. This is schematically shown in figure 3, where amplification curves of housekeeping and target

genes are depicted for an untreated control and two treated samples. Target genes generally respond to treatment with different C_t values, whereas housekeeping genes yield similar C_t values despite treatment.

In this study, the gene for the major sigma factor (σ^{70}) of *P. aeruginosa*, *rpoD*, and the gene for the malonyl-CoA-[acyl-carrier-protein]-transacylase, *fabD*, were used for normalisation. Both genes have previously been shown to be stably expressed in *P. aeruginosa* and were hence considered to be suitable reference genes (Savli et al. 2003; Potvin et al. 2008).

Various models have been described to calculate expression ratios, the delta-delta C_t and the efficiency corrected C_t model being the two most abundantly used (Livak and Schmittgen 2001; Pfaffl 2001; Fleige et al. 2006). In both methods, the deviation in C_t values, designated as ΔC_t, is calculated for both reference and target genes by subtracting the C_t value of the sample from the control (Yuan et al. 2006).

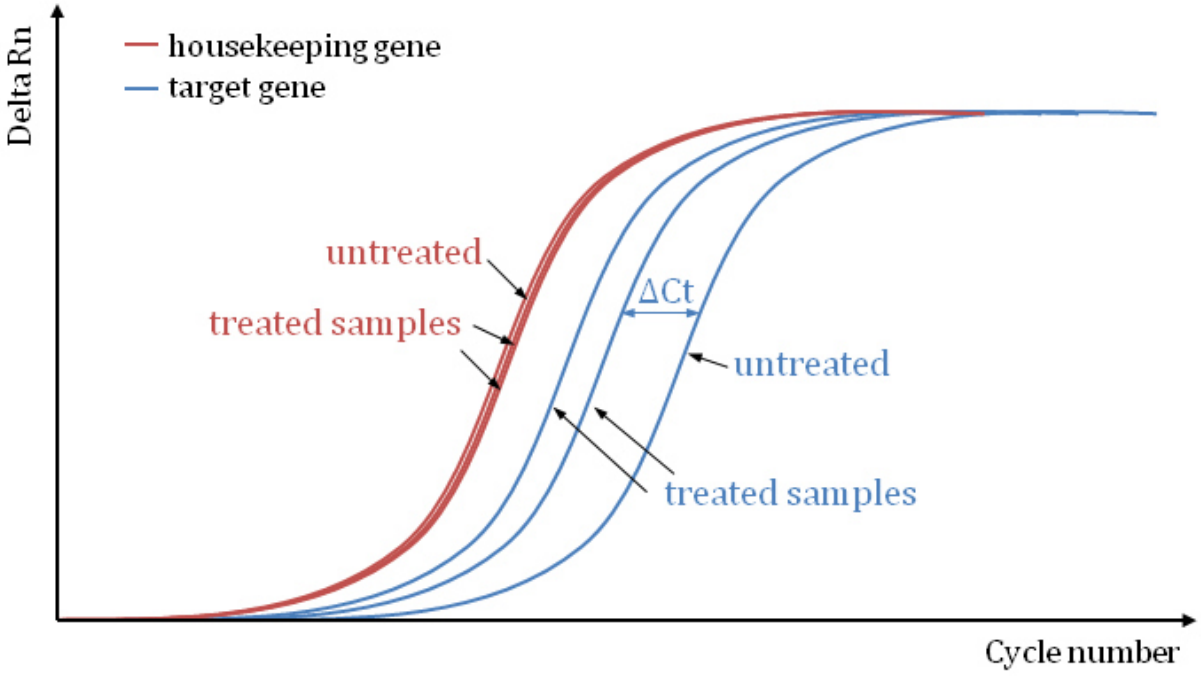

Fig. 3 Schematic diagram of amplification curves of housekeeping and target genes. Housekeeping genes are represented by red lines, whereas target genes are represented by blue lines. While target genes are differentially expressed in different treated samples compared to the untreated control, housekeeping genes remain stable in their expression. The resulting change in C_t values is indicated by ΔC_t

In the first method, no efficiency correction is performed and the equation for the calculation of an expression ratio between a treated sample and an untreated control is as follows:

$$R = 2^{-[\Delta Ct\ treated\ sample - \Delta Ct\ untreated\ control]} \qquad Equation\ 6$$

$$R = 2^{-\Delta\Delta Ct} \qquad Equation\ 7$$

where R is the relative expression ratio of a target gene based upon the C_t deviation of an unknown treated sample versus an untreated control in comparison to a reference gene. The theoretical efficiency of 100 % is indicated by 2.

The second approach incorporates specific PCR efficiencies so that equation 6 is expressed as:

$$R = \frac{(E_{target})^{\Delta Ct\ target\ (untreated\ control - treated\ sample)}}{(E_{ref})^{\Delta Ct\ ref\ (untreated\ control - treated\ sample)}} \qquad Equation\ 8$$

As the C_t value of the chosen unregulated reference gene should be the same in the untreated control and the treated sample, the ΔC_t becomes 0, so that equation 8 can be shortened to yield equation 9:

$$R = (E_{target})^{\Delta Ct\ target\ (untreated\ control - treated\ sample)} \qquad Equation\ 9$$

In the following, a calculation of relative gene expression ratios is shown exemplary with the target genes A and B for the two cultivation conditions referred to as 'treated 1' and 'treated 2' as well as the control sample, referred to as 'untreated'. The C_t values for the calculations are shown in table 3 and 4. For normalisation, the housekeeping genes *rpoD* and *fabD* were used.

First, the ΔC_t values are calculated for the target genes obtained for treated sample 1 and the untreated sample:

$$\Delta Ct\ gene\ A\ (untreated - treated\ 1) = 25.986 - 25.271 = 2.57$$

$$\Delta Ct\ gene\ B\ (untreated - treated\ 1) = 28.444 - 28.783 = -0.34$$

In the same way, the ΔC_t values are calculated for the reference genes *fabD* and *rpoD*:

$$\Delta Ct\ fabD\ (untreated - treated\ 1) = 29.653 - 29.457 = 0.196$$

$$\Delta Ct\ rpoD\ (untreated - treated\ 1) = 25.465 - 25.772 = -0.307$$

Table 3 C_t values of the housekeeping genes *fabD* and *rpoD*

Detector & Task	Sample	Ct values	Average Ct
fabD REFERENCE	untreated	29.90	29.65
		29.40	
	treated 1	31.64	29.46
		31.27	
	treated 2	33.00	29.63
		33.07	
rpoD REFERENCE	untreated	25.51	25.47
		25.42	
	treated 1	26.79	25.77
		26.76	
	treated 2	27.50	25.90
		27.31	

For the two housekeeping genes, *fabD* and *rpoD*, a mean of -0.056 is then determined for the ΔC_t values. Consequently, the fold-change in expression of the genes A and B in the treated sample 1 compared to the untreated sample is calculated according to equation 8:

$$R(gene\ A) = \frac{\left(E_{gene\ A}\right)^{\Delta Ct\ gene\ A\ (untreated - treated\ 1)}}{\left(E_{ref}\right)^{\Delta Ct\ ref\ (untreated - treated\ 1)}} = \frac{(1.99)^{2.57}}{(2.01)^{-0.056}} = 6.12$$

$$R(gene\ B) = \frac{\left(E_{gene\ B}\right)^{\Delta Ct\ gene\ B\ (untreated-treated\ 1)}}{\left(E_{ref}\right)^{\Delta Ct\ ref\ (untreated-treated\ 1)}} = \frac{(1.96)^{-0.34}}{(2.01)^{-0.056}} = 0.82$$

Table 4 C_t values of target genes, designated as *gene A* and *gene B*

Detector & Task	Sample	Ct values	Average Ct
gene A TARGET	untreated	25.97	25.99
		26.00	
	treated 1	23.34	23.41
		23.49	
	treated 2	25.36	32.072
		25.19	
gene B TARGET	untreated	28.45	28.44
		28.44	
	treated 1	28.57	28.78
		29.00	
	treated 2	30.83	32.44
		30.47	

Likewise, the ΔC_t values for the treated sample 2 are calculated first for the target genes A and B:

$$\Delta Ct\ gene\ A\ (untreated - treated\ 2) = 25.986 - 32.072 = -6.089$$

$$\Delta Ct\ gene\ B\ (untreated\ - treated\ 2) = 28.444 - 32.44 = -3.996$$

Subsequently, the ΔC_t values are calculated for the reference genes *fabD* and *rpoD*:

$$\Delta Ct\ fabD\ (untreated\ - treated\ 2) = 29.653 - 29.625 = 0.028$$

$$\Delta Ct\ rpoD\ (untreated\ - treated\ 2) = 25.465 - 25.902 = -0.437$$

Expression ratios for gene A and B in 'treated 2' are then calculated in relation to the untreated sample:

$$R(gene\ A) = \frac{\left(E_{gene\ A}\right)^{\Delta Ct\ gene\ B\ (untreated - treated\ 2)}}{\left(E_{ref}\right)^{\Delta Ct\ ref\ (untreated\ -treated\ 2)}} = \frac{(1.99)^{-6.089}}{(2.01)^{-0.205}} = 0.01$$

$$R(gene\ B) = \frac{\left(E_{gene\ B}\right)^{\Delta Ct\ gene\ B\ (untreated - treated\ 2)}}{\left(E_{ref}\right)^{\Delta Ct\ ref\ (untreated - treated\ 2)}} = \frac{(1.96)^{-3.996}}{(2.01)^{-0.205}} = 0.07$$

The obtained expression ratios can then be plotted in a bar chart diagram (Fig. 4).

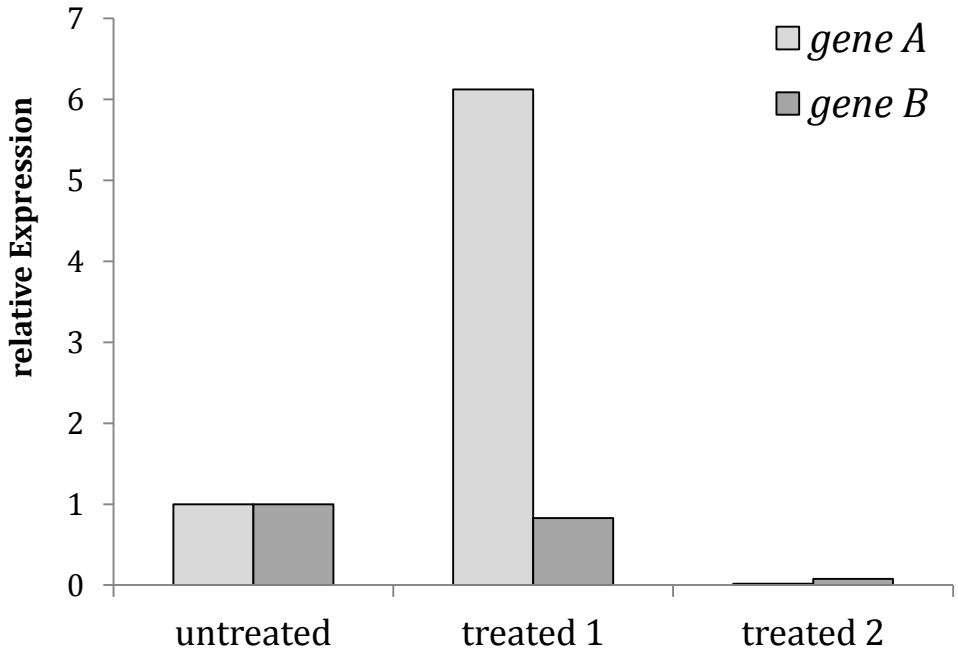

Fig. 4 Relative expression of the target genes A and B. The expression of genes A and B was normalised to gene expression of the housekeeping genes *rpoD* and *fabD*. The relative expressions are shown as bars under different cultivation conditions, designated as samples 'treated 1' and 'treated 2'. The control sample, designated as 'untreated' was used as a reference for the calculation of expression ratios

The fold-change of the control sample invariably assumes the value of 1, as the ΔC_t value equals zero and any number raised by the exponent 0 is 1. In the chosen example, gene A is strongly up-regulated compared to the untreated sample by approximately 6-fold, whereas it is strongly down-regulated in the treated sample 2 by more than 99 %. Gene B on the other hand, is neither up- nor down-regulated in the treated sample 1 compared to the untreated sample and its expression nears that under untreated conditions. In treated sample 2 on the other hand, gene B is strongly down-regulated by more than 90 %.

Apart from the demonstrated calculations, a third-party software, namely Qbase Plus (BioGazelle, Zwijnaarde, Belgium) was used for further calculation of gene expression ratios throughout the entire thesis for all calculations. This software for the management and automated analysis of qPCR data is based on the efficiency corrected delta-delta C_t model and its algorithms are implemented in the programme (Hellemans et al. 2007).

ii. Quantitative Real-Time PCR for Gene Activity Measurements in *Pseudomonas aeruginosa* PAO1 under different Cultivation Conditions

In the following chapter, the potential of quantitative PCR (qPCR) to adequately and reliantly analyse gene expression of crucial points of regulation in bioprocesses was investigated in different cultivation scenarios.

For this purpose, cultures of *P. aeruginosa* PAO1 were grown in shake flasks using standard BM2 medium, which served as the control sample for the determination of relative expression. Consequently, different cultivation scenarios were set up.

First, the influence of cell density on the expression of rhamnolipid synthesis genes was investigated by cultivating *P. aeruginosa* PAO1 in BM2 medium under different cell densities, measured as optical densities (OD), while assessing the relative gene expression for the two synthesis genes, *rhlA* and *rhlC*. Furthermore, modified BM2 media with reduced iron, nitrogen, and carbon concentrations were prepared to investigate the influence of media composition and nutrient limitation on rhamnolipid synthesis. Both carbon and nitrogen were reduced by half or one-tenth compared to standard BM2, whereas iron was completely omitted. As nitrogen source, ammonium was used in concentrations of 7 mM as in standard BM2, whereas 3.5 mM and 0.7 mM ammonium was used in the modified BM2 preparation. Glucose (0.4 %) served as carbon source in standard BM2, whereas 0.2 % and 0.04 % glucose was added in modified BM2 medium. Consequently, C_t values that is threshold cycles were determined by qPCR and relative expression for genes involved in rhamnolipid synthesis and regulation thereof was determined. The genes under investigation included the two genes for mono- and dirhamnolipid synthesis, *rhlA* and *rhlC*, as well as central components of the three quorum sensing (QS) systems of *P. aeruginosa*, i.e. the *las*, *rhl*, and *pqs* systems. Furthermore, various sigma factors were investigated, namely the genes for the stationary phase sigma factor, *rpoS*, as well as the gene for the nitrogen and iron limitation sigma factors, *rpoN* and *pvdS*, respectively. Additionally, the quantity of mono- and dirhamnolipids was measured in the cell-free culture supernatant to verify the molecular data obtained by qPCR. Additionally, gfp reporter assays were performed under equal cultivation conditions using *rhlA_gfp* and *rhlC_gfp* vectors constructs

transformed into *P. aeruginosa* PAO1 for online *in-situ* expression analyses of rhamnolipid synthesis. Furthermore, the behaviour of the two rhamnolipid synthesis genes in response to varying nitrogen sources, namely ammonium, urea, and nitrate, was under investigation.

Influence of cell density on *rhlA* and *rhlC* expression of *P. aeruginosa*

Besides nutrient limitations, cell density-dependent QS has been shown to have major implications on rhamnolipid synthesis. In order to evaluate the impact on gene expression of the two rhamnolipid synthesis genes, *rhlA* and *rhlC*, *Pseudomonas aeruginosa* PAO1 was grown in standard BM2 medium at different cell densities. In doing so, an overnight culture was used to prepare cultures with increasing ODs. Those cultures were then grown for another 3 hours before samples were taken for gene expression analyses. The ODs ranged from 0.25, which served as a reference for the calculation of gene expression ratios up to 1.6.

Gene expression analyses revealed that expression of both *rhlA* and *rhlC* did not increase until a cell density of 0.8 (Fig 1). Their expression remained as low as in cultures with the lowest investigated OD in this particular experimental set-up that is 0.25. However, in cultures with an OD of 0.9 and higher, *rhlA* started to show increased expression. At an OD of 0.9, *rhlA* was increased by 2-fold, at the next higher OD of 1.4 it was expressed almost 4-fold. The increase became even more pronounced in cultures with higher ODs, such as 1.6, where *rhlA* was expressed 10-fold. *RhlC* expression on the other hand did not show such a strong correlation with cell density. It was not increased in all cultures and its expression remained as low as in cultures with an OD of 0.25.

To summarise, the expression of *rhlA* was shown to be increased with increasing cell densities, whereas *rhlC* expression was not affected by increasing cell densities.

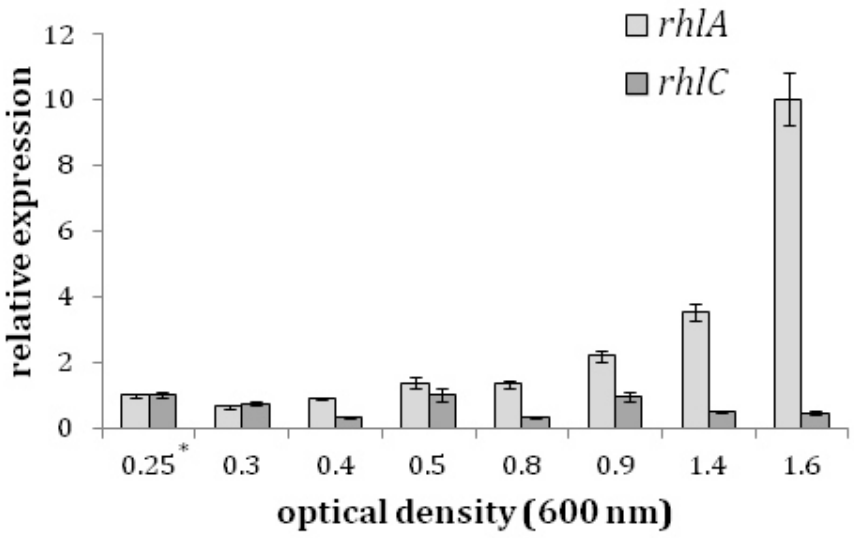

Fig. 1 Cell density-dependent expression of the two rhamnolipid synthesis genes of *P. aeruginosa* PAO1, *rhlA* and *rhlC*. Gene expression of *rhlA* and *rhlC* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as bars during shake flask experiments using standard BM2 medium with different optical densities. Samples were prepared by adjusting the particular OD and sampling took place after another 3 hours of cultivation. Expression of genes in standard BM2 medium with the lowest OD of 0.25 was used as a reference for the calculation of expression ratios and is indicated by the asterisk

Influence of nutrient limitation on *rhlA* and *rhlC* expression of *P. aeruginosa* PAO1

In reaction to nitrogen reduction from 7 mM to 0.7 mM, expression of *rhlA* was up-regulated by 10-fold, whereas *rhlC* was not up-regulated (Fig. 2b). Reduction of nitrogen to 3.5 mM, as well as carbon reduction, did not have an effect on neither *rhlA* nor *rhlC* expression and the expression levels remained as low as under standard conditions. Iron depletion, that is iron omission from the medium, on the other hand, caused a strong up-regulation of *rhlA* by more than 16-fold, whereas *rhlC* was only marginally up-regulated by a factor of 2.

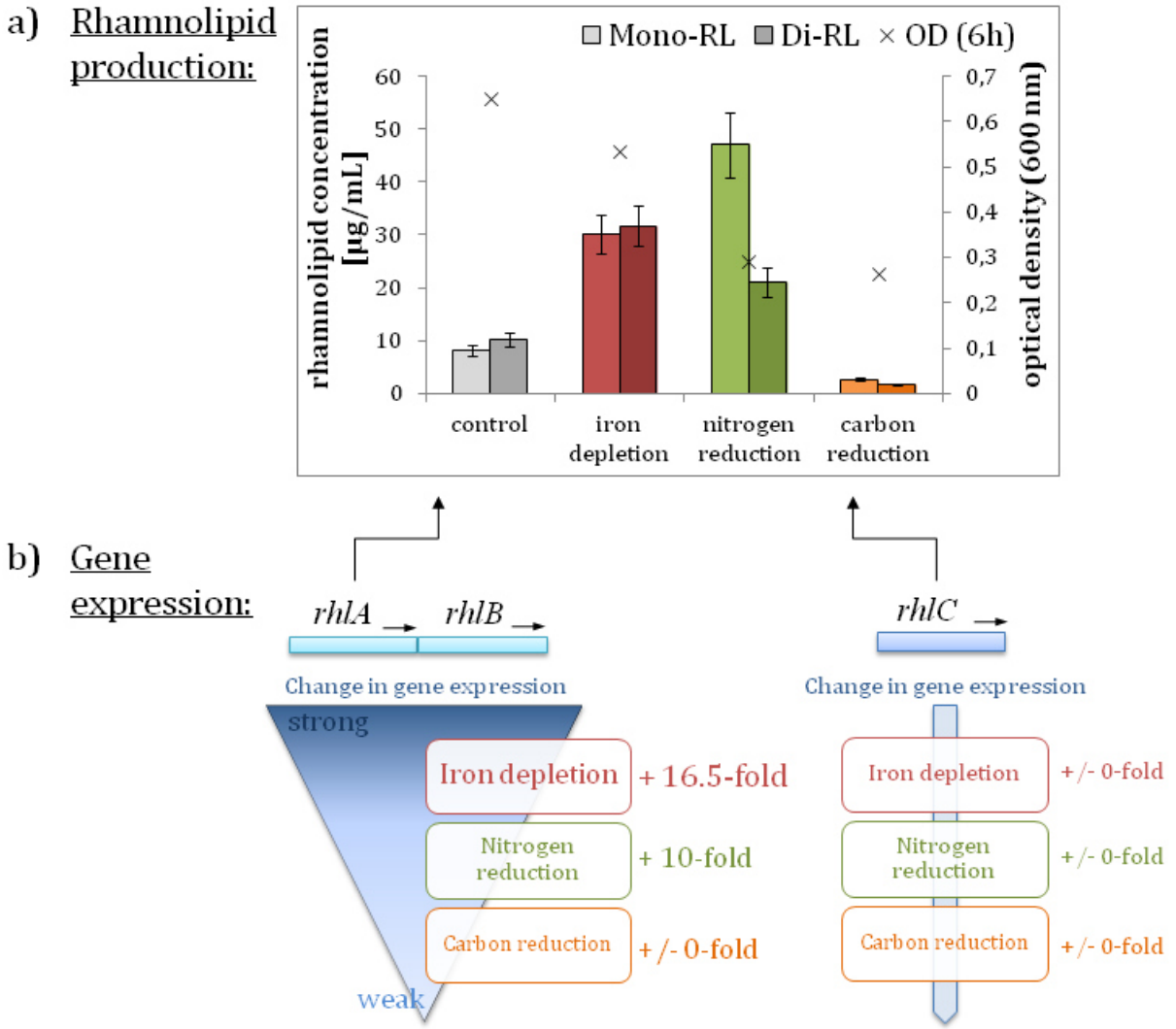

Fig.2 Influence of nutrient variation on rhamnolipid production and expression of the genes for the rhamnosyltransferases, *rhlA* and *rhlC*, *in P. aeruginosa* PAO1. Mono- and dirhamnolipids were quantified in the cell-free supernatant of shake flask cultures of *P. aeruginosa* PAO1 with different compositions of standard BM2 medium (a). Change in gene expression was assessed for the cells of the same cultures and is shown as *fold*-change under the particular cultivation conditions i.e. nitrogen and carbon reduction, and iron depletion (b). Gene expression of *rhlA* and *rhlC* was normalised to *rpoD* and *fabD* gene expression

Additionally, culture supernatants were analysed for rhamnolipid production using liquid chromatography tandem mass spectrometry (LC/MS/MS) to verify and complement the results obtained on transcription level. In the supernatant of the culture where ammonium was reduced to 0.7 mM, production of both mono- and

dirhamnolipids was strongly increased compared to standard conditions that is by factors of almost 6 and 2, respectively (Fig. 2a). Iron omission did also lead to an increase of mono-rhamnolipids by a factor of almost 4 compared to standard conditions, while dirhamnolipids were increased by a factor of 3. Carbon reduction did not lead to an increased amount of rhamnolipids and their concentration was even decreased compared to standard conditions.

To further verify the results obtained for the relative gene expression, gfp reporter assays were developed and performed under equal cultivation conditions using a *rhlA_gfp* as well as a *rhlC_gfp* vector construct transformed into *P. aeruginosa* PAO1.

Under iron deplete conditions, *rhlA_gfp* expression was strongly increased, whereas *rhlC_gfp* expression was less strongly increased (Fig. 3a and b). Reduction of nitrogen or carbon did not cause an up-regulation of neither *rhlA_gfp* nor *rhlC_gfp* expression.

Gene expression analyses of cultures of *P. aeruginosa* PAO1 in shake flasks revealed that both genes for rhamnolipid synthesis, *rhlA* and *rhlC*, were differentially expressed in response to varying media compositions, i.e. nitrogen, iron, and carbon reduction. The results obtained for the relative gene expression could be verified by both gfp reporter assays and rhamnolipid quantification in the culture supernatant. This demonstrated the ability of the chosen system to reliantly reflect changes in the regulatory network on transcriptional level with *rhlA* and *rhlC* expression as the most crucial read-out concerning rhamnolipid production.

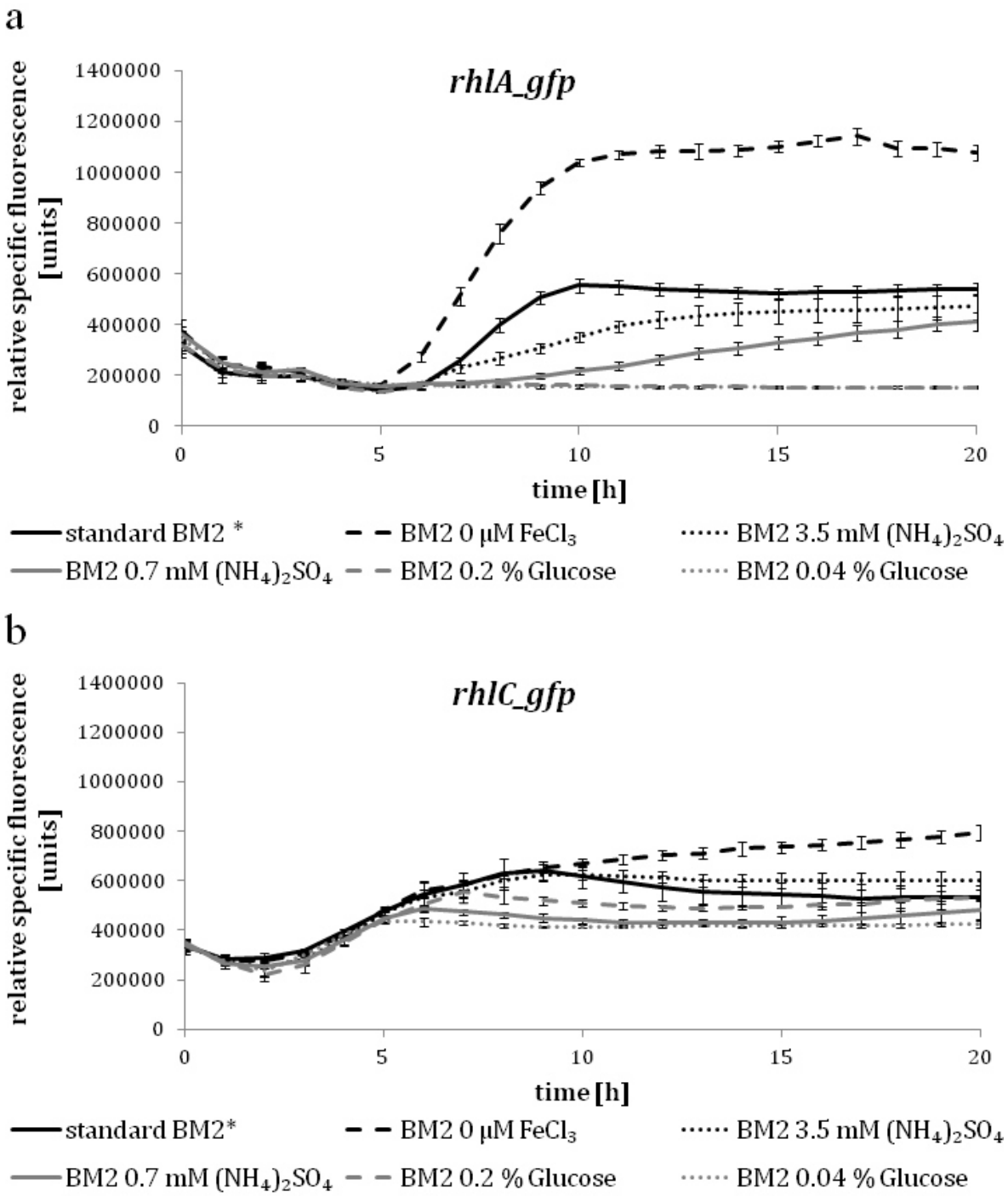

Fig. 3 Expression of *rhlA_gfp* (a) and *rhlC_gfp* (b) in planktonic cultures of *P. aeruginosa* PAO1 in response to various nutrient offers. Standard BM2 medium (10 µM $FeCl_3$; 7 mM $(NH_4)_2SO_4$; 0.4 % glucose) is represented by a solid black line and marked by the asterisk. Dashed black line represents cultures grown in standard BM2 medium without addition of $FeCl_3$. Nitrogen reduced BM2 medium with 3.5 mM $(NH_4)_2SO_4$ is represented by a dotted black line, whereas 0.7 mM $(NH_4)_2SO_4$ is represented by a solid grey line. Carbon reduced BM2 medium with 0.2 % glucose is represented by a dashed grey line and BM2 with 0.2 % glucose is represented by a dotted grey line. Measured gfp emission was correlated to the measured optical density at 600 nm to produce specific gfp emission

Influence of nutrient limitation on the QS and regulatory network of rhamnolipid synthesis of *P. aeruginosa* PAO1

In order to be able to monitor and fully comprehend the complex regulatory network behind rhamnolipid synthesis, central components of the QS systems, as well as sigma and regulatory factors were investigated under the same limiting conditions as described above using qPCR.

The *las* system, represented by the gene for the transcriptional regulator, *lasR*, was very strongly up-regulated in response to carbon reduction to 0.04 % glucose that is almost 16-fold compared to standard conditions (Fig. 4). The gene for the corresponding autoinducer synthase, *lasI*, on the other hand was not up-regulated under neither carbon nor nitrogen reduction. Nitrogen reduction to 0.7 mM ammonium however, led to an increased expression of both *lasI* and *lasR* by 2- and 3-fold respectively. In response to iron omission, only *lasR* expression was slightly increased by almost 2- fold.

The *rhl* system responded more strongly to nutrient reduction (Fig. 4). Under carbon, and nitrogen reduction to 0.04 % glucose and 0.7 mM ammonium, respectively, as well as under iron omission, the gene for the transcriptional regulator, *rhlR*, was increased by almost 4-fold. The gene for the C4-HSL synthase, *rhlI*, was only slightly up-regulated by 2-fold under carbon, nitrogen, and iron reduction. For the *pqs* system, a similar pattern could be observed (Fig. 4). In response to carbon reduction to 0.04 % glucose and nitrogen reduction and iron omission, the gene for the response regulator, *mvfR*, was up-regulated by almost 5-fold in the case of carbon reduction and almost 3-fold in the case of nitrogen and iron reduction.

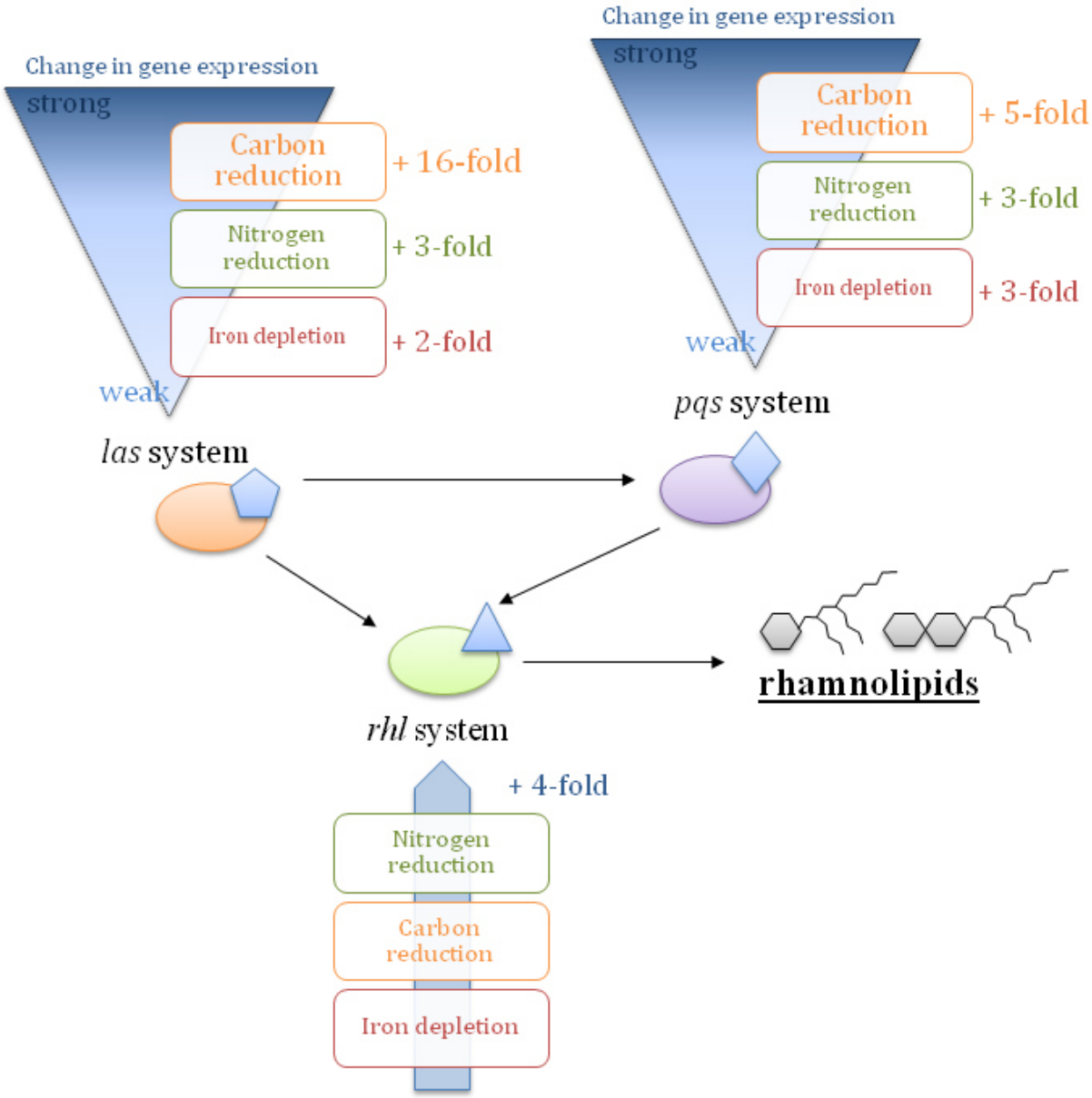

Fig. 4 Influence of nutrient limitations on the gene expression of components of the three QS systems in *P. aeruginosa* PAO1. Change in gene expression is shown as *fold*-change under the particular cultivation conditions i.e. nitrogen and carbon reduction, and iron depletion. Gene expression of *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsE*, and *mvfR* was normalised to *rpoD* and *fabD* gene expression

The gene for the sigma factor for nitrogen limitation, *rpoN*, as well as the gene for the stationary growth phase sigma factor, *rpoS*, was found to be strongly up-regulated under carbon reduction to 0.04 % glucose, i.e. 8- and 10-fold, respectively (Fig. 5). Both genes were also up-regulated in response to nitrogen reduction, however only approximately

3-fold. Iron omission did not affect the expression of neither *rpoN* nor *rpoS*. *PvdS*, the gene for the sigma factor for iron limitation, however was strongly up-regulated under iron deplete conditions by almost 40-fold (Fig. 5). Both carbon and nitrogen reduction did not have an up-regulatory effect on *pvdS* expression.

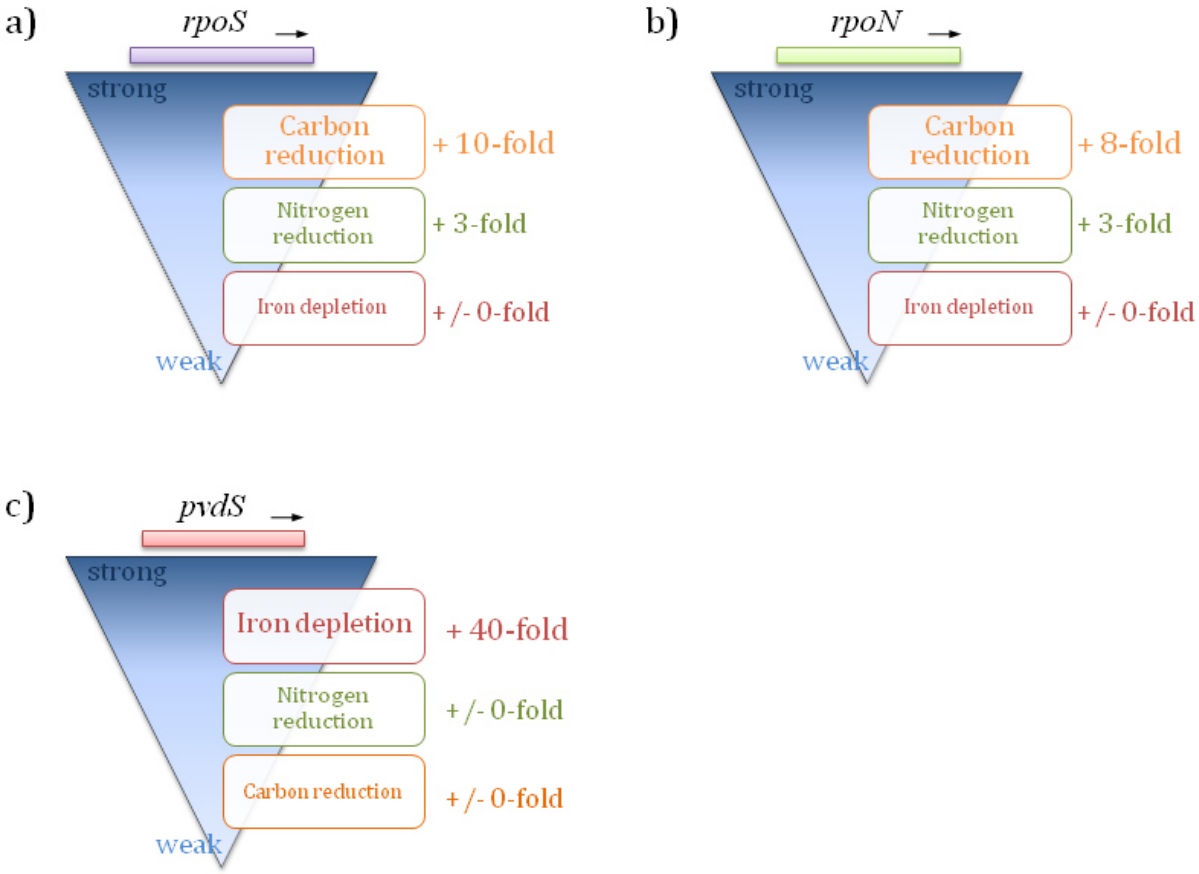

Fig. 5 Influence of nutrient limitation on the expression of the for the sigma factors, *rpoS* (a), *rpoN* (b), and *pvdS* (c) of *P. aeruginosa* PAO1. Change in gene expression is shown as *fold*-change under the particular cultivation conditions i.e. nitrogen and carbon reduction, and iron depletion. Gene expression of *rpoS*, *rpoN*, and *pvdS* was normalised to *rpoD* and *fabD* gene expression

The two enzymes for rhamnolipid biosynthesis, *rhlA* and *rhlC* were successfully characterised in response to different cultivation conditions in the first part of this chapter. Likewise, the greater regulatory network can be monitored by the molecular toolbox, as gene expression analyses of various factors associated with the regulation of

rhamnolipid production, i.e. QS components or sigma factors, demonstrated in the second part of this chapter.

The established molecular toolbox was hence considered potent to be used to describe bioprocesses on transcriptional level and that the data obtained can be used to identify new strategies for process optimisation.

Influence of different nitrogen sources on *rhlA* and *rhlC* expression of *P. aeruginosa* PAO1

In the previous section, differential gene expression in response to nutrient limitations was shown using qPCR. Consequently, further experiments were set-up to assess the effect of different nitrogen sources on rhamnolipid synthesis. Besides ammonium, which is the nitrogen source in standard BM2, nitrate and urea were chosen. Both gene expression of *rhlA* and *rhlC* as well as rhamnolipid production in the supernatant were under investigation.

After 7 hours of cultivation, neither nitrate nor urea caused an up-regulation of rhamnolipid synthesis genes (Fig. 6a). The expression of both genes, especially *rhlA*, was even down-regulated in response to urea as nitrogen source. However, after 24 hours of cultivation, expression of both *rhlA* and *rhlC* was strongly increased by 6-fold in cultures, where nitrate served as nitrogen source (Fig. 6b). Urea on the other hand did not cause an up-regulation compared to standard BM2 after 24 hours of cultivation and *rhlA* and *rhlC* expression remained as low as under standard conditions with ammonium as nitrogen source.

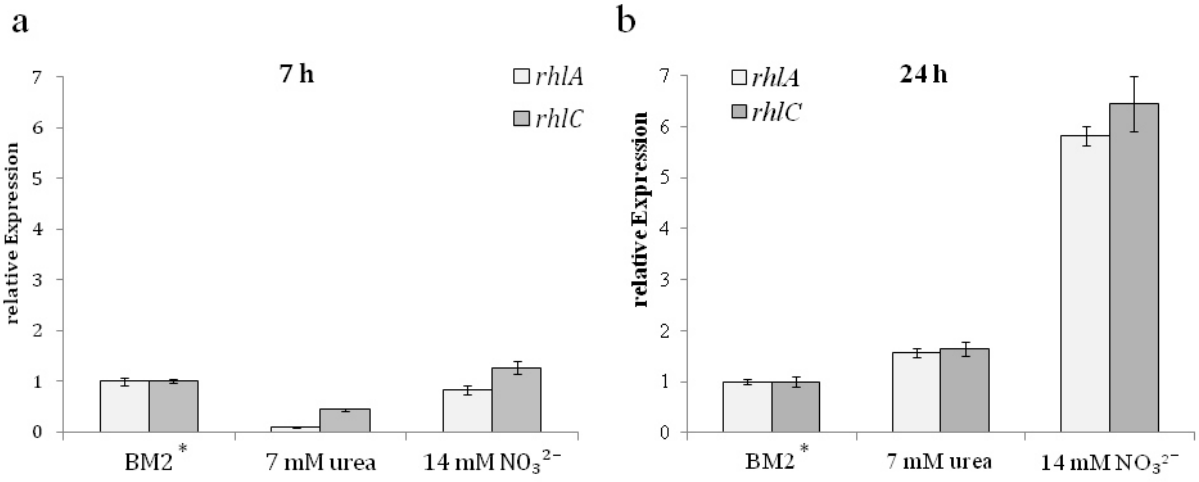

Fig. 6 Relative gene expression of rhamnosyltransferases 1 and 2 (*rhlA* and *rhlC,* respectively) in *P. aeruginosa* PAO1 in response to different nitrogen sources. The gene expression of *rhlA* and *rhlC* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as bars during shake flask experiments using standard BM2 medium with different nitrogen sources. Samples were collected during mid-exponential phase after 7 (a) and 24 hours (b) of cultivations. Expression of genes in standard BM2 medium (10 µM $FeCl_3$; 7 mM $(NH_4)_2SO_4$; 0.4 % glucose) was used as a reference for the calculation of expression ratios and is indicated by the asterisk

In the culture supernatants, mostly monorhamnolipids were increasingly produced in cultures with nitrate as nitrogen source that is approximately 4-fold more compared to the amount produced under standard conditions (Fig. 7). In cultures with urea as nitrogen source, almost twice as much monorhamnolipids was produced compared to that in BM2. Dirhamnolipids were generally less produced. Approximately the same amount of dirhamnolipids was produced in cultures, where nitrate and ammonium served as nitrogen sources, whereas cultures with urea produced half the amount compared to these conditions. Taken together, cultures with nitrate as nitrogen source produced twice as many mono- and dirhamnolipids, i.e. total rhamnolipids, than cultures with urea or ammonium.

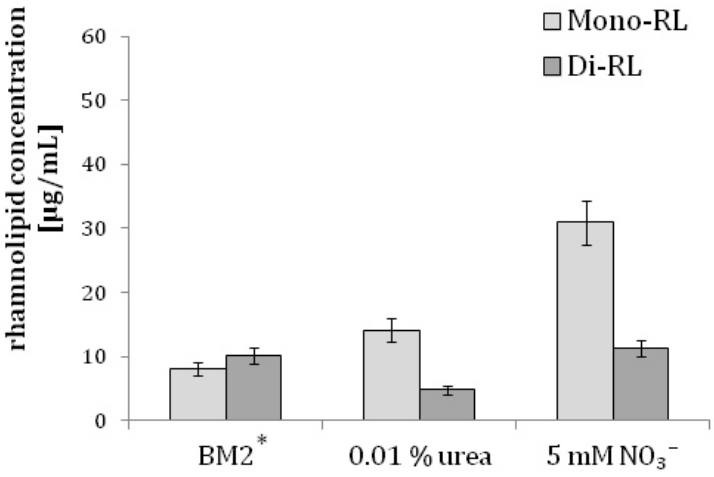

Fig. 7 Rhamnolipid production of *P. aeruginosa* PAO1 in response to different nitrogen sources. Mono- and dirhamnolipids were quantified in the supernatant of cultures of *P. aeruginosa* PAO1 grown 24 hours in shake flasks with BM2 and different sources of nitrogen using LC/MS/MS. BM2 medium is considered as a reference with 10 µM $FeCl_3$; 7 mM $(NH_4)_2SO_4$; 0.4 % glucose and indicated by the asterisk

To summarise, amongst the investigated nitrogen sources, nitrate seems to influence rhamnolipid production most positively. Both the expression of the rhamnolipid synthesis genes as well as the production of rhamnolipids in the supernatant were increased in those cultures with nitrate as nitrogen source compared to cultures that were grown with ammonium or urea.

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iii. Expression of Genes involved in Rhamnolipid Synthesis in *Pseudomonas aeruginosa* PAO1 in a Bioreactor Cultivation

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Expression of genes involved in rhamnolipid synthesis in *Pseudomonas aeruginosa* PAO1 in a bioreactor cultivation

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Abstract There is a growing demand for economic bioprocesses based on sustainable resources rather than petrochemical-derived substances. Particular attention has been paid to rhamnolipids—surface-active glycolipids—that are naturally produced by *Pseudomonas aeruginosa*. Rhamnolipids have gained increased attention over the past years due to their versatile chemical and biological properties as well as numerous biotechnological applications. However, rhamnolipid synthesis is tightly governed by a complex growth-dependent regulatory network. Quantitative comprehension of the molecular and metabolic mechanisms during bioprocesses is key to manipulating and improving rhamnolipid production capacities in *P. aeruginosa*. In this study, *P. aeruginosa* PAO1 was grown under nitrogen limitation with sunflower oil as carbon and nitrate as nitrogen source in a batch fermentation process. Gene expression was monitored using quantitative PCR over the entire time course. Until late deceleration phase, an increase in relative gene expression of the *las*, *rhl*, and *pqs* quorum-sensing regulons was observed. Thereafter, expression of the rhamnolipid synthesis genes, *rhlA* and *rhlC*, as well as the *las* regulon was downregulated. *RhlR* was shown to remain upregulated at the late phase of the fermentation process.

Keywords *Pseudomonas aeruginosa* PAO1 · Rhamnolipid · Biosurfactant · Gene expression · Batch cultivation · Quorum sensing

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Introduction

Rhamnolipids are surface-active glycolipids that are produced by several *Pseudomonas* species, as well as bacteria from other families, classes, or phyla (Abdel-Mawgoud et al. 2010). However, rhamnolipid production remains a characteristic for *Pseudomonas aeruginosa* (Rahman et al. 2010). Among the *P. aeruginosa* species, PAO1 is the best-studied producer yet with a fully sequenced genome (Stover et al. 2000; Winsor et al. 2009).

Rhamnolipids produced by *P. aeruginosa* have gained attention as they represent an alternative to petrochemical surface-active components (Banat et al. 2000). Whilst rhamnolipids were discovered more than 60 years ago by Jarvis and Johnson (1949), industrial production has not yet been achieved in large scale, which is mainly due to low product yields, excess foaming, relatively costly raw materials, and expensive downstream processing (Banat et al. 2000). Additionally, the complex regulatory network behind rhamnolipid synthesis in *P. aeruginosa* constitutes a major obstacle in the way to upscale production, especially when it comes to expression in heterologous nonproducing hosts. Attempts with recombinant *Escherichia coli* (Cabrera-Valladares et al. 2006) or *Pseudomonas putida* (Cha et al. 2008) resulted in relatively low product yields that were far below those achieved by its natural producer. In 2011, Wittgens et al. have demonstrated rhamnolipid production using the genetically optimised *P. putida* KT24C1 pVLT31_*rhlAB* grown on glucose as carbon source (Wittgens et al. 2011).

The highest yields so far have been reported by Giani et al., who claims to have developed a process that uses *P. aeruginosa* strains DSM 7107 and DSM 7108 to produce yields between 70 and 120 g/L in their patent from 1997 (US5501966). However, no such yield was reported on laboratory scale ever since, which makes it even more crucial to fully understand the regulatory mechanisms behind rhamnolipid synthesis in *P. aeruginosa*.

Synthesis of monorhamnolipids in *P. aeruginosa* is mediated by the rhamnosyltransferase subunit A, RhlA, which catalyses the synthesis of fatty acid dimers that subsequently serve as the precursor for the rhamnosyltransferase 1 subunit B (RhlB) to form monorhamnolipids and synthesis of dirhamnolipids is catalysed by the rhamnosyltransferase 2 (RhlC; Rahim et al. 2001; Deziel et al. 2003; Soberon-Chavez et al. 2005). The genes for rhamnosyltransferase 1, namely *rhlA* and *rhlB*, are encoded on one single operon, the *rhlAB* regulon, and are hence co-expressed (Sullivan 1998). Both the *rhlAB* operon and the *rhlC* gene are under the control of growth-dependent quorum-sensing (QS), and rhamnolipid production has been shown to be directly linked to the *rhl* QS system via the regulator RhlR (Medina et al. 2003c). However, several other factors beside cell density seem to influence *rhlAB* as well as *rhlC* expression and it has become evident that the entire QS system itself and hence rhamnolipid synthesis are subjected to additional layers of regulation (Schuster and Greenberg 2006), as most QS-controlled genes show a pattern of delayed expression and so does *rhlAB*. The *rhlAB* operon is also partially dependent on the alternative sigma factor RpoS (σ^{38} or σ^{S}; Medina et al. 2003b). Other studies link rhamnolipid synthesis to nutritional conditions, such as nitrogen exhaustion and the alternative sigma factor for nitrogen limitation, RpoN (σ^{54}), has been shown to be involved in the regulation of *rhlAB*, as well as *rhlR* and *rhlC* expression (Rahim et al. 2001; Medina et al. 2003a, c). However, the *rhlAB* promoter itself has been demonstrated to be independent of RpoN. The dependency of *rhlAB* expression on this sigma factor might occur indirectly through RhlR, whose gene harbours a RpoN-dependent transcriptional start site (Medina et al. 2003c). This allows coordinate expression and hence production of dirhamnolipid only when its precursors, monorhamnolipids, are present. Several other QS-related and unrelated transcriptional, as well as posttranscriptional regulation mechanisms that influence rhamnolipid synthesis have been described and are reviewed by Reis et al. (2011). Other important regulatory factors involved in rhamnolipid synthesis that have been under investigation during the presented batch fermentation are the genes for RsaL, GacA, AlgR, LipC, and QscR. The *rsaL* gene is activated by the *las* regulon and codes for the regulatory protein RsaL. It is believed to provide QS homeostasis and indirectly affect rhamnolipid synthesis (Rampioni et al. 2006, 2007). So far, the environmental stimuli that activate *rsaL* expression are unknown (Schuster and Greenberg 2006; Reis et al. 2011). GacA is part of the GacA/GacS two-component system that was shown to antagonise the small RNA-binding protein RsmA. RsmA in turn negatively regulates the QS system and directly activates rhamnolipid synthesis (Heurlier et al. 2004). Furthermore, GacA has been shown to positively affect *lasR*, *rhlR*, and *rhlI* transcription (Pessi et al. 2001). The alginate biosynthesis regulatory protein AlgR has been reported to negatively regulate the *rhl* system and *rhlAB* expression (Morici et al. 2007). Furthermore, the lipase LipC, has been described to positively affect rhamnolipid production (Rosenau et al. 2010). Quorum-sensing control repressor (QscR) on the other hand is a cell density-dependent QS regulator homologue, which represses different QS-activated genes through antagonistic actions (Schuster and Greenberg 2006; Oinuma and Greenberg 2011).

Recently, another layer to QS control has been described, that is "quorum quenching", which is a mechanism for the disruption of the quorum-sensing signalling circuitry through cleavage of AHL autoinducers by acylases or lactonases (Hong et al. 2012). Two potent mechanisms for quorum quenching have been identified in bacteria, that is destabilising the LuxR family protein receptors for AHL molecules (de Kievit and Iglewski 2000) and by degrading AHL signals through lactonases (Lin et al. 2003) or acylases (Huang et al. 2003, 2006; Sio et al. 2006; Wahjudi et al. 2011). So far, three acylases have been identified in *P. aeruginosa*, namely PvdQ, HacB, and QuiP, all of which are able to cleave the long chain AHL autoinducer C12-HSL (Huang et al. 2006; Sio et al. 2006; Wahjudi et al. 2011; Hong et al. 2012). The acylase PvdQ has recently been reported to have not only quorum-quenching functions, but is also involved in iron homeostasis and is only expressed under iron starvation (Ochsner et al. 2002; Lamont and Martin 2003; Nadal Jimenez et al. 2010). PvdQ was demonstrated to decrease C12-HSL levels at low iron concentrations and *PvdQ* mutants of *P. aeruginosa* PA14 grown under iron-limiting conditions showed impairment in biofilm formation and swarming motility, as well as reduced virulence (Nadal Jimenez et al. 2010).

So far, batch fermentations constituted a molecular black box in regard to transcriptional activity of the genes of the rhamnolipid synthesis circuitry. In this study, we present a molecular biology-based strategy for understanding the regulation of rhamnolipid synthesis during batch fermentation. This innovative approach focuses on generating expression profiles of different factors that have previously been described to be involved in the regulation of rhamnolipid synthesis. Monitoring gene expression over the entire time course of a batch fermentation provides pivotal information about regulatory events during rhamnolipid synthesis in such a process. The method for generating the expression profiles was quantitative polymerase chain reaction (PCR), which

allows the assessment of gene expression at the most dynamic level. For the presented batch fermentation, *P. aeruginosa* PAO1 was grown in a nitrogen-limited production medium with 125 g/L sunflower oil as sole carbon and nitrate as nitrogen source.

Materials and methods

Bacterial strains and culture methods

P. aeruginosa PAO1 (DSM 22644) was used for the batch fermentation, as well as the planktonic cultures (Müller et al. 2010). Cells were grown in BM2 medium for 24 h at 37 °C in a rotary shaker (MaxQ 4000, Thermo Scientific) at 170 rpm in shaking flasks allowing optimal aeration. BM2 medium was composed of a 10 × phosphate ammonia base containing 0.07 M $(NH_4)_2SO_4$, 0.4 M K_2HPO_4 and 0.22 M KH_2PO_4, as well as 40 % glucose, 200 mM $MgSO_4 \times 7\ H_2O$ and 10 mM $FeSO_4 \times 7\ H_2O$. All components were sterilised by autoclaving, except for $FeSO_4$, which was filter sterilised using a 0.2-μm membrane filter (VWR, Karlsruhe, Germany). Nitrogen-limiting conditions were achieved by using one half or one tenth of the ammonium sulphate concentration that is used in standard BM2. For the batch fermentation in a 42-L bioreactor, *P. aeruginosa* PAO1 was grown in a nitrogen-limited production medium with 125 g/L sunflower oil as sole carbon source. The medium was furthermore supplemented with a Ca^{2+}-free mineral salt solution containing 15 g/L $NaNO_3$, 0.5 g/L $MgSO_4 \times 7\ H_2O$, 1 g/L KCl, and a 0.1 M sodium phosphate buffer. Cultivations were conducted at pH 6.5, whereas 4 M NaOH or 4 M H_3PO_4, respectively, were used to adjust pH levels. Additionally, 1 mL/L trace element solution was added in total. The trace element solution was composed of 2.0 g/L sodium citrate $\times 2\ H_2O$, 0.28 g/L $FeCl_3 \times 6\ H_2O$, 1.4 g/L $ZnSO_4 \times 7\ H_2O$, 1.2 g/L $CoCl_2 \times 6\ H_2O$, 1.2 g/L $CuSO_4 \times 5\ H_2O$, and 0.8 g/L $MnSO_4 \times H_2O$. After preparation, the solution was filter sterilised with a 0.22-μm membrane filter (Carl Roth GmbH, Karlsruhe, Germany). The batch fermentation was according to Müller et al. (2011).

Preparation of seed cultures for batch fermentation

Precultures of *P. aeruginosa* PAO1 were prepared from glycerol stocks using a total volume of 100 μL stock solution in 25 mL Luria–Bertani (LB). Overnight cultivation was conducted at 37 °C in 250 mL baffled shake flasks at 120 rpm (Multitron II, HT Infors, Bottmingen, Switzerland). Prior to batch fermentation, 5 mL of the preculture were inoculated in 200 mL production medium supplemented with 25 g ($\delta_{\text{sunflower oil}}$=0.921 g/mL) sunflower oil at 37 °C in 1 mL baffled shake flasks at 120 rpm. After 24 h of incubation, an appropriate volume of this culture was used to inoculate the bioreactor. For this purpose, a theoretical OD_{580} of 0.06 was used.

Production of rhamnolipids using *P. aeruginosa* PAO1 in 30-L bioreactor scale

For batch fermentation, a 42-L stirred tank bioreactor, with a nominal working capacity of 30 L was used (Biostat® Cplus, Sartorius Stedim Biotech, Göttingen, Germany). The bioreactor featured an integrated process control system for pH, temperature, minimal dissolved oxygen (pO_2), airflow, and foam level regulation (MFCS/win 2.1, Sartorius BBI, Göttingen, Germany). Cultivation was conducted at 37 °C under a constant pO_2 of 5 % at a stirring rate of 400 rpm. The bioreactor was equipped with a Rushton turbine for dispersion of air bubbles and mixing, propeller-type stirrers for axial mixing, and a specifically designed foam stirrer to ensure homogeneity of the suspension. pO_2 was controlled by varying the aeration rate between 0.1 and 3.3 L/ (L×min). To control excess foaming, a mechanical foam breaker was installed in the headspace of the bioreactor. Trace element solution was added four times over the whole fermentation process, namely at the start of cultivation, as well as 20, 40, and 70 h during cultivation. Furthermore, pH level was controlled and if necessary adjusted to pH 6.5 using 4 M NaOH or 4 M H_3PO_4 respectively. The batch fermentation was according to Müller et al. (2011).

Sampling

Samples for offline molecular analysis were taken approximately every 5–6 h, starting at 8.5 h after sufficient biomass accumulation. Gene expression ratios were then calculated relatively to this first time point of sampling. RNA stabilisation was conducted on-site using RNAProtect® Bacteria Reagent from Qiagen (Hilden, Germany) according to manufacturer's instructions. Samples were stored at −80 °C until sample preparation. Furthermore, samples for offline analysis of biodry mass (BDM), sunflower oil, mono-/dirhamnolipid and nitrate concentration were routinely taken as described by Müller et al. 2011. Briefly, culture broth was mixed with equal volumes of *n*-hexane to remove excess sunflower oil. Subsequently, mass of sunflower oil was obtained gravimetrically from the organic phase. Rhamnolipid quantification was done as described previously (Müller et al. 2011). Biomass concentration was obtained gravimetrically from three samples, with the highest relative error of 13.8 %. The concentration of rhamnolipid was determined by HPLC analysis, with a maximum error of 7.1 %.

RNA isolation and reverse transcription

Total RNA was isolated after RNA stabilisation by phenol/chloroform extraction using Qiagen's QIAzol® Lysis Reagent in combination with the RNeasy® Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was subsequently digested using TURBO® DNase (Invitrogen, Darmstadt, Germany) and successful digestion was then evaluated by using polymerase chain reaction followed by loading eventual products on an agarose gel. To control RNA integrity as well as quality, 260/280 and 260/230 ratios were assessed photospectrometrically (NanoDrop® ND-1000, Thermo Scientific, Frankfurt, Germany) and isolated RNA was additionally loaded on agarose gels. RNA concentrations were then measured and a total of 250 ng RNA was used for cDNA synthesis. For each sample, an equal amount of RNA was transcribed into cDNA. Random-primed reverse transcription was conducted using MultiScribe® Taqman Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. The temperature profile was according to protocol (25 °C, 10 min; 48 °C, 30 min; 95 °C, 5 min; 4 °C, ∞). After reverse transcription, samples were directly used for real-time PCR or otherwise stored at −20 °C.

Gene expression analysis using quantitative PCR

Gene expression was assessed using a SYBR Green mediated two-step quantitative real-time PCR. Prior to PCR, RNA was first transcribed into cDNA as described above and in a second step, the quantitative real-time PCR was performed using an ABI PRISM® 7300 (Applied Biosystems) in combination with KAPA® SYBR FAST qPCR ABI PRISM (PEQLAB, Erlangen, Germany). Primers used for real-time PCR are listed in Table 1. Prior to gene expression analyses, primers were checked for appropriate product amplification and primer efficiencies were determined. All primers used met the requirements as well as the tolerable efficiency range of 90–110 %.

Table 1 Primers for quantitative real-time PCR

Primer or target	Sequence	Source or reference	Amplicon size (bp)
algR	F 5′-ATCAGTGCACGGACCCGCAA-3′ R 5′-GCATGGCGCAAGGTCACGTA-3′	This study	95
fabD	F 5′-GCATCCCTCGCATTCGTCT-3′ R 5′-GGCGCTCTTCAGGACCATT-3′	Savli et al. (2003)	163
gacA	F 5′-ATATCAGCCCGCAGATCGCCCA-3′ R 5′-TTCGGCGACAGGCACAGCTT-3′	This study	166
lasI	F 5′-GCCCCTACATGCTGAAGAACA-3′ R 5′-CGAGCAAGGCGCTTCCT-3′	Schwartz et al. (2007)	62
lasR	F 5′-GTGGAGCGCCATCCTGCAGA-3′ R 5′-CGGTCGTAATGCTCGCGCCA-3′	This study	144
lipC	F 5′-ACTACACCCGCACGCGCTAT-3′ R 5′-GTTGCTGTTCACCGGCGACT-3′	This study	155
pqsE	F 5′-GCGCTTGAACCGGCAACTGT-3′ R 5′-GGCGTCGCACGTCGTAGAAA-3′	This study	185
pvdS	F 5′-AGATGTGGTCCAGGATGCGT-3′ R 5′-GTGTTCGAGGGTCGCGTAGT-3′	Viducic et al. (2007)	232
qscR	F 5′-AAGCAATGTGCGCCTGACCG-3′ R 5′-TTCACCGTGCGCTGGTCGAT-3′	This study	111
rhlA	F 5′-GATCGAGCTGGACGACAAGTC-3′ R 5′-GCTGATGGTTGCTGGCTTTC-3′	This study	95
rhlC	F 5′-ATCCATCTCGACGGACTGAC-3′ R 5′-GTCCACGTGGTCGATGAAC-3′	Neilson et al. (2010)	159
rhlI	F 5′-AGCTGGGACGCTACCGGCAT-3′ R 5′-TGGCGGCTCATGGCGACGAT-3′	This study	136
rhlR	F 5′-GAGGAATGACGGAGGCTTTTTG-3′ R 5′-CTTCTTCTGGATGTTCTTGTGG-3′	Kayama et al. (2009)	255
rpoD	F 5′-GGGCGAAGAAGGAAATGGTC-3′ R 5′-CAGGTGGCGTAGGTGGAGAA-3′	Savli et al. (2003)	178
rpoN	F 5′-ACCCGTAGTAGTGGATGGTGC-3′ R 5′-TATGGCCTGTTGCAGCTGCG-3′	Bazire et al. (2005)	128
rpoS	F 5′-CTCCCCGGGCAACTCCAAAAG-3′ R 5′-CGATCATCCGCTTCCGACCAG-3′	Savli et al. (2003)	198
rsaL	F 5′-AGCCCCAAAACATGGCCTTCCG-3′ R 5′-CTCGAAACGACTGCCGCAGGAT-3′	Lee et al. (2011)	113

Data analysis

Gene expression data was obtained using the ABI PRISM® 7300 associated SDS Software (v1.4, Applied Biosystems) and subsequently analysed using the Qbase Plus software from BioGazelle (Zwijnaarde, Belgium), which calculates relative gene expression based on the delta-delta Ct method (Pfaffl 2001; Hellemans et al. 2007). The Ct value represents the cycle number, at which the amount of amplified DNA, i.e., fluorescence passes the threshold that is background fluorescence. The Ct value is inversely proportional to the quantity of RNA/cDNA (Goni et al. 2009). As reference genes for relative quantification calculations served the housekeeping genes the major sigma factor σ^{70}, *rpoD*, and the malonyl-CoA-[acyl-carrier-protein]-transacylase *fabD*, which have previously been shown to be stably expressed in *P. aeruginosa* and are hence considered suitable as reference genes (Savli et al. 2003; Potvin et al. 2008). Regarding the expression profiles, equal amounts of cDNA were used for each sample and the relative gene expression was then calculated using the delta-delta Ct method. In doing so, the expression of a particular target gene at a certain time point during the fermentation was compared with the expression of the same target gene at 8.5 h. This expression ratio was then normalised against the two reference genes. Therefore, the expression of all target genes of interest at 8.5 h is considered to be one.

For the analysis of biomass growth, a fourth-degree polynomial equation was used to fit the data. For the analysis of rhamnolipid production, a logistic equation with four parameters was used to fit the data. The formula used for the logistic growth is described by Müller et al. (2010). Calculations were done using a scientific data analysis and graphing software (SigmaPlot 9.0, Systat, San José, USA). Specific growth rate (μ) and maximum specific growth rate (μ_{max}) were calculated based on the derivation of the polynomial fitting. Analogue to this, the specific production rate of total rhamnolipids per BDM ($q_{RL,\ spec}$) was calculated based on the BDM and rhamnolipid fittings. Concentrations of total rhamnolipids were obtained by adding concentrations of mono- and dirhamnolipids.

Results

P. aeruginosa PAO1 was grown in a nitrogen-limited production medium with 125 g/L sunflower oil as carbon and nitrate as nitrogen source in a batch fermentation.

During the batch process, biodry mass formation showed logistic behaviour, although, biodry mass began to decline at the end of the process after 49 h of fermentation (Fig. 1a). Concentrations of both mono- and dirhamnolipids showed similar logistic behaviour, i.e., the rhamnolipid production started to increase after 20 h and reached a plateau after approximately 45 h at the end of the fermentation (Fig. 1b). Dirhamnolipids reached higher concentrations as monorhamnolipids. The μ_{max} was reached after approximately 4 h fermentation. This early stage of fermentation from 0 to 4 h was therefore termed "acceleration phase" (phase I; Fig. 2). The stage after this growth rate maximum was then referred to as 'deceleration phase' due to the observed decrease in growth rate (phase II; Fig. 2). After approximately 41 h, the specific growth rate became negative and the stage after this time point was hence termed "lysis phase" (phase III; Fig. 2). The specific rhamnolipid production rate (q_{RL}) was at a maximum after 22 h, which corresponds with mid-deceleration phase.

Figure 3 gives an overview over the normalised Ct values of all reference and target genes during the fermentation process. This gives indirect information about the transcriptional activity of the genes of interest, as high Ct values correlate with low transcription levels and vice versa. Distribution of Ct values provides further information, if and how much a certain gene is regulated during the bioprocess, as Ct values for less regulated genes lie closer together. Whereas most target genes are expressed at similar levels, *rhlA* in particular showed much higher amounts of transcript than the gross of target genes, whose Ct values range between 20 and 24. However, Ct values for *rhlA* already appear between 13 and 15 and show a wide range of 13–22. *PqsE* on the other hand, as well as *lipC* and *qscR* seem to produce fewer transcripts with Ct values ranging between 30 and 26. Other genes, whose Ct values also showed a broad distribution, were *rhlC* and *rhlR*.

Gene expression of *rhlA* was strongly increased during the first 25 h of fermentation and showed an upregulation of up to 420-fold during early deceleration phase compared to its expression at the beginning of the fermentation (Fig. 4a). The expression of the *rhlC* gene was also increased, but not as much as *rhlA*. During deceleration phase, *rhlC* showed an increase in expression of up to 16-fold (Fig. 4b). *RhlA* expression reached a climax of 530-fold and *rhlC* expression of 20-fold after 41 h of cultivation at the end of the deceleration phase. Thereafter, that is after transition into lysis phase, *rhlA* expression dropped down by a factor of more than 3, whereas *rhlC* expression decreased by a factor of 1.2 (Fig. 4a and b).

In order to comprehend the mechanisms that lead to a change in expression of the genes for rhamnolipid synthesis and hence rhamnolipid production over the time course of the bioprocess, further gene expression analyses were performed. First, central quorum-sensing components of the *las*, *rhl*, and *pqs* systems were monitored.

The expression of the transcriptional regulator gene *rhlR* was increased as the culture entered deceleration phase and was found to be upregulated ten times after the first 15 h of

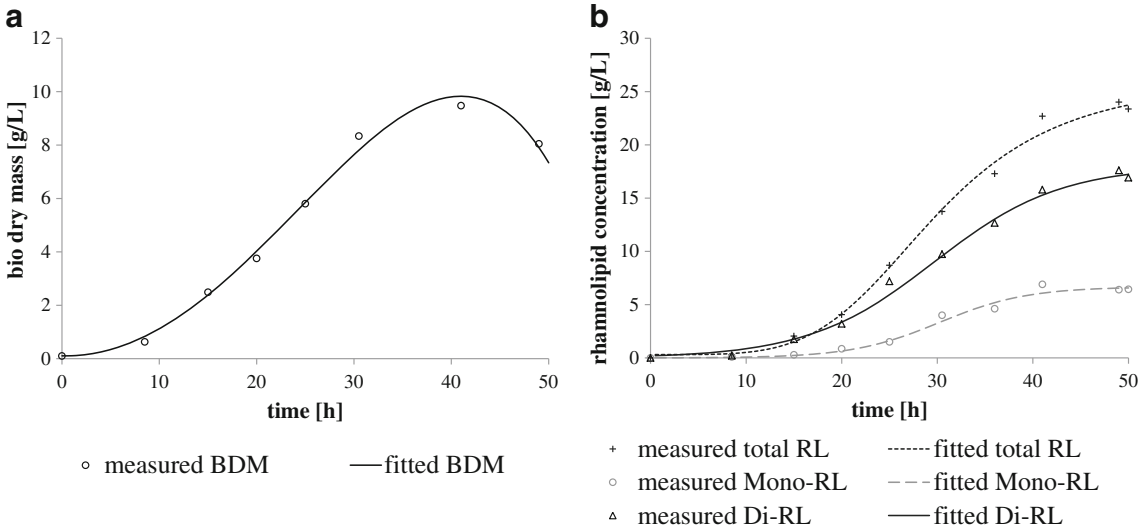

Fig. 1 Biodry mass formation and rhamnolipid production by *P. aeruginosa* PAO1 during batch fermentation. Biodry mass is represented by *black circles*. The curve for the logistic fit of biodry mass formation is shown as *black line*. Measured concentrations of total rhamnolipids were calculated by adding measured concentrations of mono- and dirhamnolipid and are shown as *black plusses*. The curve for the logistic of total rhamnolipid concentrations is shown as *dotted black* line. Measured concentrations of dirhamnolipids are shown as *black triangles*, whilst monorhamnolipids are represented by *grey circles*. The curve for the logistic fit for dirhamnolipid concentrations is shown as *black line* and for monorhamnolipids, it is shown as *dashed grey line*

fermentation. Thereafter, expression increased and reached a maximum of almost 19-fold after 41 h (Fig. 5). *RhlR* expression remained high during lysis phase at a level of about 18-fold. The gene of the cognate autoinducer synthase for C4-HSL, *rhlI*, was less strongly expressed. During deceleration phase, the relative *rhlI* expression was twice to four times higher than at acceleration phase (Fig. 5). Its expression steadily increased until it reached a maximum of 6.5-fold after 41 h of cultivation at the end of growth. Thereafter, the relative expression of *rhlI*, dropped down by half.

The *las* system generally showed low relative expression compared to the reference at 8.5 h (Fig. 6a). The gene for the transcriptional regulator, *lasR*, showed a somewhat different trend than its homologue, *rhlR*. Expression of *lasR* was upregulated by a factor of 3 after 15 h at the beginning of the deceleration phase, but after that, its expression was neither up- nor downregulated significantly ensuing from its expression at the start of fermentation (Fig. 6a). However, by the end of the deceleration phase, *lasR* expression was increased again by almost twofold compared to 8.5 h and increased further to a relative expression level of 2.5-fold after 41 h. In the lysis phase, its expression dropped down by half compared to the expression level at the previous time point. Expression of the gene for the 3-oxo-C12-HSL synthase, *lasI*,

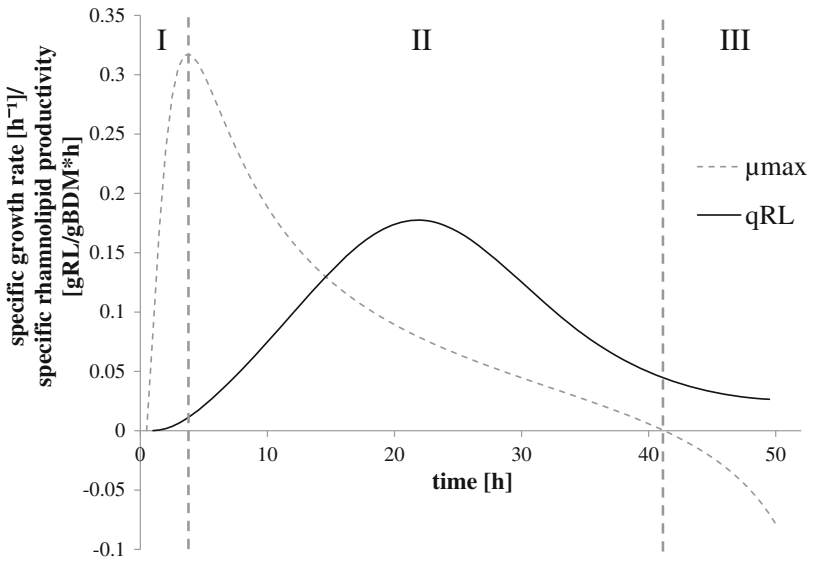

Fig. 2 Specific growth rate and specific rhamnolipid productivity by *P. aeruginosa* PAO1 during batch fermentation. The specific growth rate (μ_{max}) is shown as *dashed light grey line* and specific rhamnolipid productivity of total rhamnolipids (q_{RL}) is shown as *black line*. *I* acceleration phase, *II* deceleration phase, *III* lysis phase

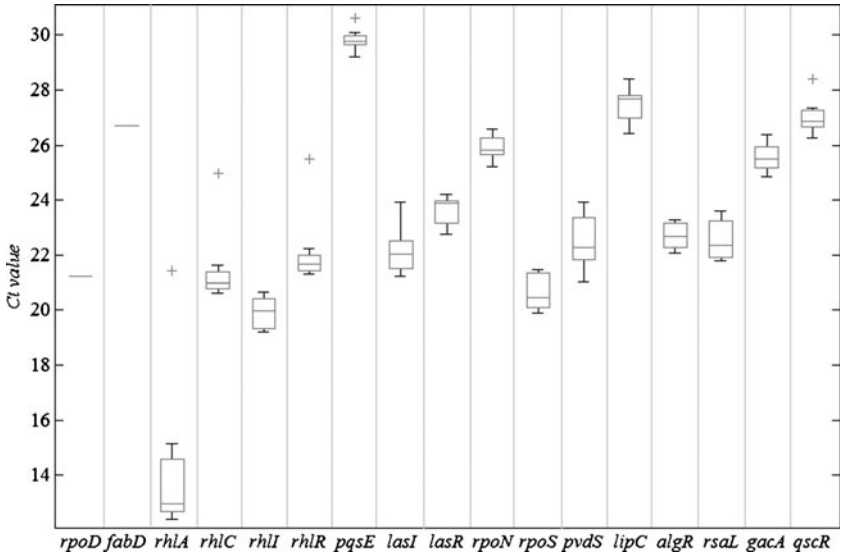

Fig. 3 Ct values of reference genes and target genes. Each *box* represents all Ct values that were measured for one target gene over the entire time course of the fermentation and were later normalised against the two reference genes *rpoD* and *fabD*. Outliers are indicated by *plus signs*. As expression of the two reference genes is considered to be constant, they appear as *horizontal lines* in the box plot diagram

on the other hand reached a temporary expression maximum with a twofold upregulation at 20 h (Fig. 6a). Thereafter, *lasI* expression levels dropped down to values comparable to the reference point, that is to say an expression of approximately one. After 36 and 41 h of fermentation, at the end of growth, the relative *lasI* expression was found to be increased again and was twice as much compared to the reference at 8.5 h. During lysis phase, *lasI* expression decreased by a factor of 2 based on its level at the previous time point. For monitoring the *pqs* system, expression of *pqsE*, referred to as quinolone signal response protein, was assessed. *PqsE* expression doubled during the first 20 h of fermentation compared to time point zero and more or less stagnated thereafter (Fig. 6b). At the end of the deceleration phase after 41 h of cultivation, it was upregulated with a relative expression of almost fourfold higher compared to the beginning of the process. After that, the relative expression of *pqsE* dropped by a factor of approximately 1.5 based on the previous time point.

Furthermore, expression of various sigma factors was investigated. The expression of the gene for the stationary phase sigma factor, *rpoS*, steadily increased during the deceleration phase starting from an expression level of 1 after 15 h, reaching a level of approximately 3 after 36 h and maintaining this level until the end of the deceleration phase at 41 h (Fig. 7a). In the lysis phase, expression of *rpoS* dropped down by a factor of 3 to the same level as at the beginning of cultivation. PvdS is a sigma factor that is only expressed under iron starvation and has been shown to control expression of virulence factors, such as endoproteases and endotoxin A (Potvin et al. 2008). The gene for the iron sigma factor, *pvdS*, was upregulated by a factor of 2 at the beginning of the deceleration phase after 15 h of fermentation, but then showed a downregulation by 50 % after 20 h (Fig. 7a). By the end of the deceleration phase, *pvdS* expression was similar to *rpoS* expression with regard to the expressional trend. *PvdS* gene expression increased steadily to a

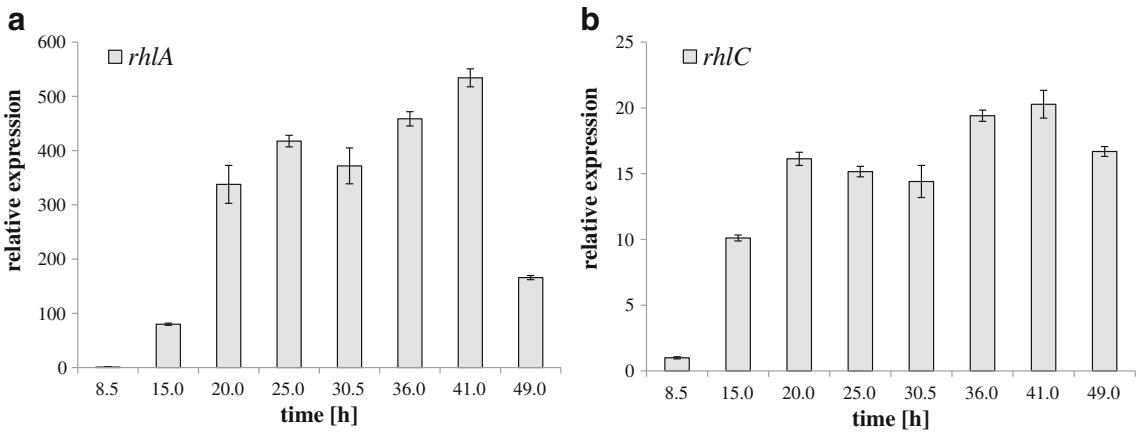

Fig. 4 Gene expression of rhamnosyltransferases 1 and 2 (*rhlA* and *rhlC*, respectively) in *P. aeruginosa* PAO1 during batch fermentation. The gene expression of *rhlA* and *rhlC* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at the specific times of fermentation

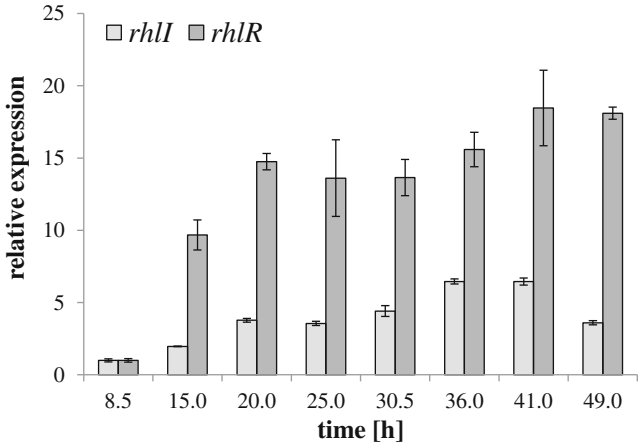

Fig. 5 Expression of the QS genes *rhlR* and *rhlI* in *P. aeruginosa* PAO1 during batch fermentation. The expression of the *rhlR* and *rhlI* genes was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at the specific times of fermentation

maximum at 41 h, where its expression level was more than thrice as high compared to the reference at 8.5 h. After entering lysis phase, *pvdS* expression decreased and dropped down by factor 7, so that its expression level was even lower than at the beginning of fermentation. *RpoN*, the gene for the sigma factor for nitrogen limitation, on the other hand, showed a different trend as *rpoS* and *pvdS*. During early deceleration phase after 15 h of fermentation, *rpoN* reached a maximum relative expression (Fig. 7b). During lysis phase after 41 h of fermentation, *rpoN* expression decreased.

Additionally, various factors already known to be involved in the regulation of rhamnolipid synthesis have been monitored to get an overview of and additional insight into the multicomponent regulatory network beyond QS.

GacA/GacS is a two-component global regulatory system that has no direct effect on rhamnolipid production, but was described to act indirectly upon rhamnolipid synthesis through RsmA (Heurlier et al. 2004). RsaL is a global regulator, that is believed to provide QS homeostasis through repressing the transcription of the gene for the autoinducer synthase LasI by coordinate binding together with LasR to the *rsaL-lasI* bidirectional promoter (Rampioni et al. 2007). During the fermentation process, the expression profiles of *gacA* and *rsaL* showed a similar trend as that of *lasR*. Both *gacA* and *rsaL* expression reached a maximum of almost threefold during early deceleration phase after 15 h (Fig. 8a). Over the next 10 h of fermentation, *rsaL* and *gacA* expression decreased and reached another maxium of five- and threefold, respectively, by late deceleration phase. After reaching lysis phase, both *rsaL* and *gacA* expression decreased. Gene expression of the RNA-binding protein RsmA was also under investigation, but *rsmA* was not seen to be regulated during the fermentation (data not shown). Lipase C is one of two secreted lipases of *P. aeruginosa* and its physiological function has not yet been described. However, it has been shown to affect both biofilm formation and rhamnolipid production in *P. aeruginosa* (Rosenau et al. 2010). Expression of the lipase *lipC* steadily increased during deceleration phase until it reached an expression level maximum of 5.5-fold after 41 h (Fig. 8b). During lysis phase, the expression dropped down by a factor of 3. The alginate biosynthesis regulatory protein *algR* has been shown to repress *rhlA* and *rhlR* expression in a biofilm-specific manner (Morici et al. 2007). During the fermentation process, *algR* expression showed a similar trend as *lipC*. After 36 h, *algR* expression was three times higher than at the starting point of the cultivation (Fig. 8c). During the lysis phase by the end of the fermentation process after 49 h, *algR* expression was already subsiding and was reduced by 30 % compared to its highest expression at 36 h. The expression profile of the quorum-sensing control repressor, QscR, fell out of alignment and showed a somewhat divergent trend, whereas

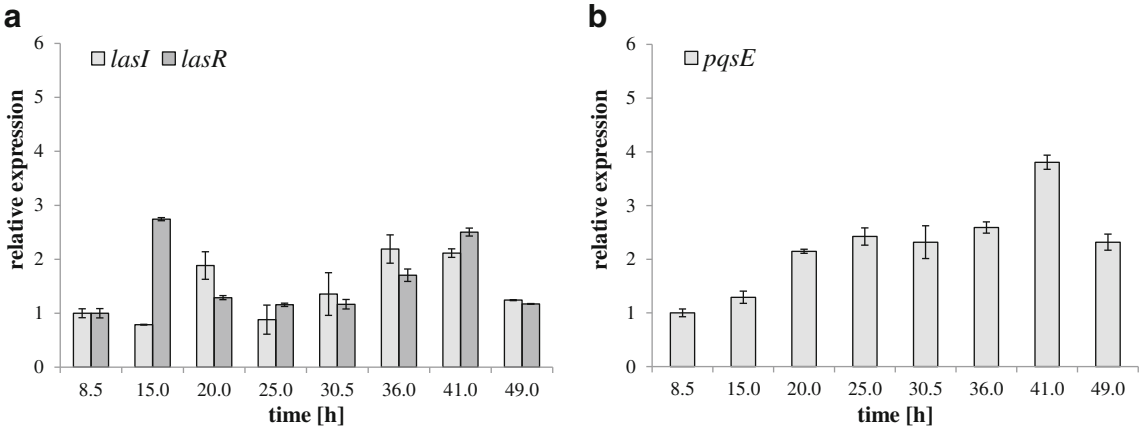

Fig. 6 Expression of the QS genes *lasR*, *lasI* and *pqsE* in *P. aeruginosa* PAO1 during batch fermentation. The expression of the *pqsE*, *lasI*, and *lasR* genes was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as bars at the specific times of fermentation

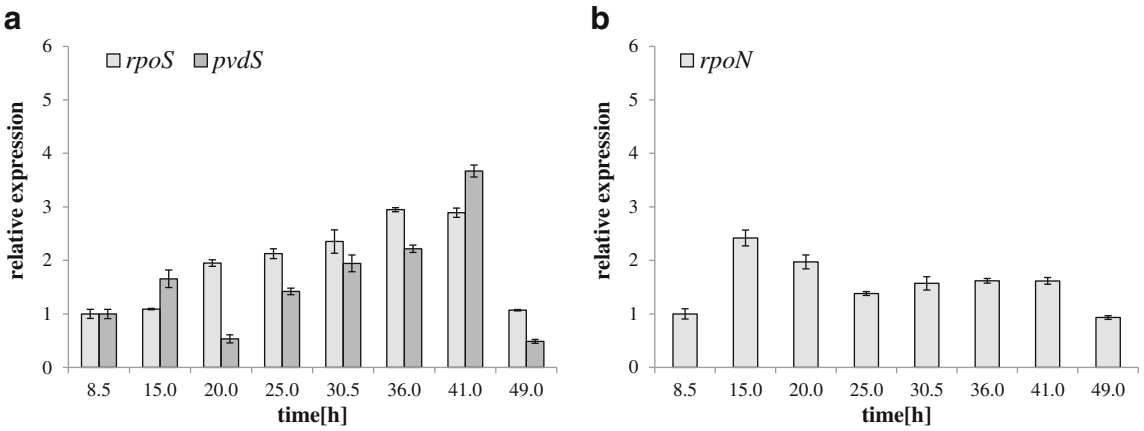

Fig. 7 Gene expression of the sigma factors RpoS, pvdS, and RpoN in *P. aeruginosa* PAO1 during batch fermentation. The expression of the *rpoN*, *rpoS*, and *pvdS* genes was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at the specific times of fermentation

an expression maximum of 5.5-fold was already reached at mid-deceleration phase after 25 h (Fig. 8d). Thereafter, expression slowly decreased to a relative expression of 2.5-fold over the course of the remaining fermentation process.

Furthermore, expression of the catabolite repressor homologue VfR and the exponential growth phase regulator DksA was monitored. However, neither *vfR* nor *dksA* were subjected to regulation and were generally downregulated during the fermentation (data not shown) compared to the reference time point at the start of the fermentation.

To investigate the capability of the chosen methodology to reliably identify new "setscrews" for manipulating the process, *P. aeruginosa* PAO1 was confronted with different compositions of the standard BM2 minimal medium. For system validation, the expression of the genes for the two rhamnosyltransferases, *rhlA* and *rhlC*, was assessed as a read-out. Exemplary, gene activity in response to nitrogen availability is shown. Under nitrogen limiting conditions, both *rhlA* and *rhlC* expression was strongly increased that is to say approximately 30- and 20-fold, respectively, compared to gene expression under standard conditions (Fig. 9). This could further be confirmed by determination of both mono- and dirhamnolipids in the culture supernatant using LC-MS-MS (data not shown). Other manipulation strategies are currently being investigated by our group and are subject to further studies.

Discussion

Rhamnolipids have increasingly become the focus of attention as an alternative to traditional ecotoxic petrochemical surface-active components (Banat et al. 2000). However, the complex growth-dependent regulatory network behind rhamnolipid synthesis in *P. aeruginosa* constitutes a major obstacle in the way to upscale production.

In this study, a molecular biology-based approach is presented for understanding the regulatory mechanisms during batch fermentation using *P. aeruginosa* PAO1 grown under nitrogen limitation with 125 g/L sunflower oil as sole carbon source, as it is currently used for the production of rhamnolipids (Müller et al. 2010). The main aim of this study is to build a basis for the characterisation of the gene regulatory network behind large-scale rhamnolipid production in a batch process in order to be able to modify and control the process on the most sensitive level–the transcriptional level. The first response of the cell to outside stimuli and most immediate adaption to changes in the surrounding happens on RNA level. Assessing changes in RNA level may also be more straightforward rather than assessing what comes at the end of a long cascade, i.e., rhamnolipid productivity. Furthermore, monitoring the cell's activity on the transcriptional level may give more accurate information about the actual state of the cell compared with conventional analytical methods for displaying the culture's status, i.e., determination of biodry mass, CO_2 exhaust, or pH levels. Based on a well-characterised gene regulatory network, strategies for selective and targeted process control can be developed. Investigating the expression of different factors involved in the regulation of rhamnolipids during batch fermentation is taking "gene regulatory stock" so to say and gives useful information about what particular system responds at what time point to which extent.

Gene expression profiling revealed that the genes for rhamnolipid synthesis, *rhlA* and *rhlC*, were strongly regulated during the fermentation process. At the end of the deceleration phase, both *rhlA* and *rhlC* reached the highest relative expression rate. After transition into lysis phase, the expression of both genes decreased relative to their highest expression. However, their genetic activity was still clearly detectable and much higher compared to expression levels at

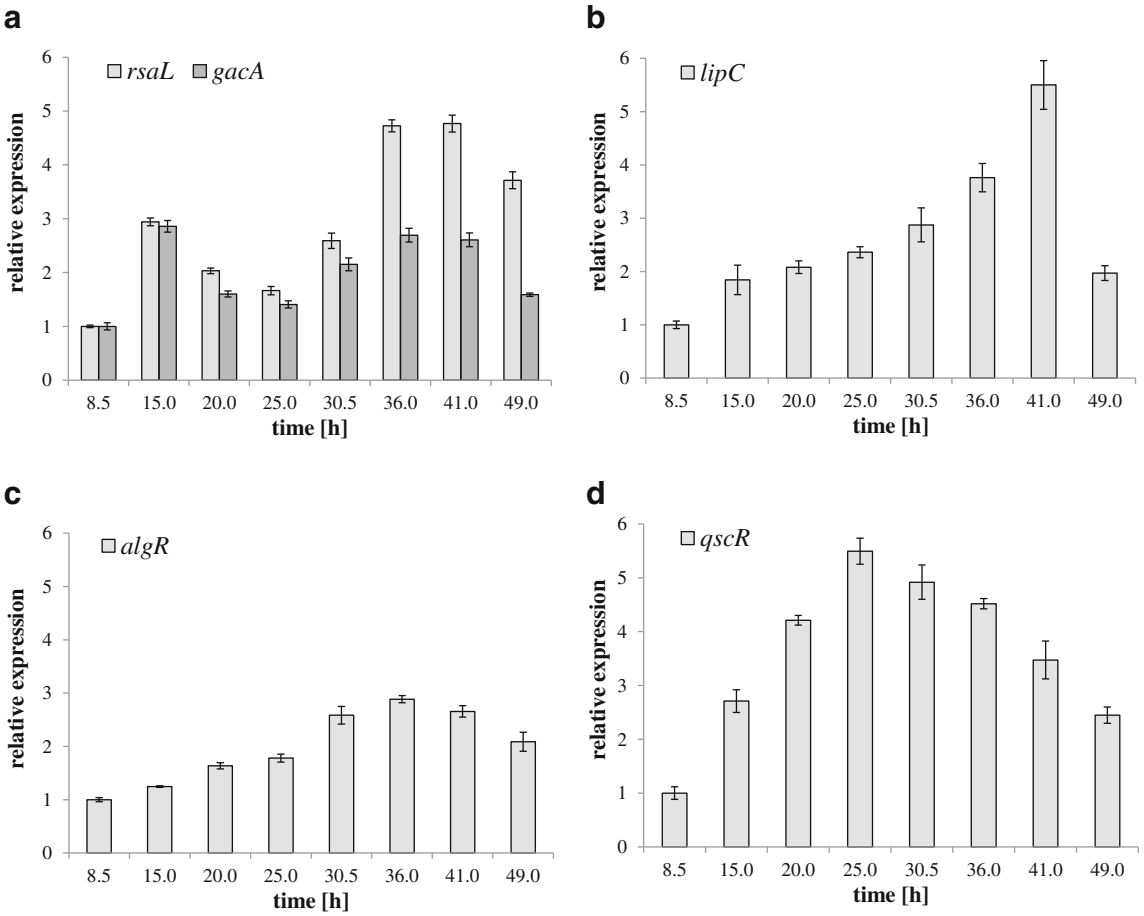

Fig. 8 Gene expression of regulatory factors in *P. aeruginosa* PAO1 during batch fermentation. The expression of the *rsaL*, *gacA*, *lipC*, *algR*, and *qscR* genes was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at the specific times of fermentation

the start of the process. The presented transcriptional results confirmed analytical data, i.e., the specific rhamnolipid productivity from previous fermentations, where *P. aeruginosa* PAO1 was grown under the same conditions as in the current study, except for the sunflower oil starting concentration, which was 250 g/L. Under these particular conditions, the specific rhamnolipid production rate was shown to have its maximum at the early stationary growth phase, after which the specific rhamnolipid productivity decreased and eventually subsided to minimal levels (Müller et al. 2010).

To investigate molecular mechanisms that might be responsible for the regulation of rhamnolipid production in the course of a batch fermentation, gene expression of QS system components of the *rhl*, *las*, and *pqs* system as well as sigma factors affected by stationary growth phase and nitrogen starvation were assessed. Additionally, other regulatory factors associated with rhamnolipid regulation were monitored to get an insight and overview of the multicomponent regulatory network beyond the central QS circuitry.

The expression of the genes for the QS factors, namely the autoinducer synthase, *rhlI*, as well as the gene for the quinolone signal response protein, *pqsE*, showed a similar expression profile as the two rhamnolipid synthesis genes. Their expression increased until late deceleration phase and related to this maximal expression decreased thereafter to lower, yet measureable levels. Expression of the gene for the transcriptional regulator, *rhlR*, increased likewise. However, *rhlR* expression remained on the same level after entering lysis phase and did not decrease. The upregulation of *rhlI* might indicate an increased potential to synthesise C4-HSL, subsequently creating an auto-inductory feedback loop upon itself (Schuster and Greenberg 2006). This is further supported by the fact that *rhlR* was found to be likewise upregulated. Consequently, this would lead to an RhlR-C4-HSL-dependent activation of *rhlAB* operon expression, resulting in increased amounts of rhamnosyltransferases and hence rhamnolipids. Previous studies also have shown that other regulatory factors are influencing the transcriptional *rhlR* regulation of *P. aeruginosa* (Heurlier et al. 2003; Croda-Garcia et al. 2011). Here, the virulence factor regulator (Vfr) directly regulated the *rhlR* transcription via binding to promoter regions. This

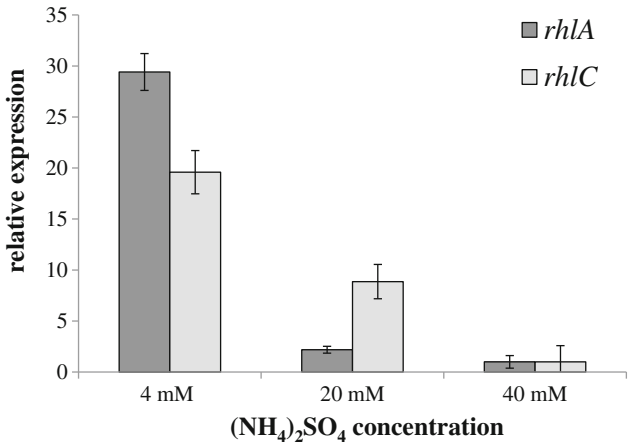

Fig. 9 Expression of the two rhamnosyltransferase genes, *rhlA* and *rhlC*, in *P. aeruginosa* PAO1. The gene expression of *rhlA* and *rhlC* was normalised to *rpoD* gene expression. The relative expressions are shown as *bars* under different concentrations of ammonium sulphate in shake flask experiments. The concentration of ammonium sulphate in standard BM2 equals 40 mM and is considered as control and reference for the calculation of relative gene expression

interaction together with increased LasR concentration has led to increased expression of *rhl*R by a positive feedback autoregulation. Therefore, the observed rhamnolipid production during deceleration growth phase of the fermentation process might be due to Vfr regulation, which needs further investigation.

Another way to regulate the QS-mediated rhamnolipid synthesis is by "quorum quenching" (Hong et al. 2012). The known *P. aeruginosa* acylases, PvdQ, HacB, and QuiP, are able to cleave the long-chain autoinducer C12-HSL (Huang et al. 2006; Sio et al. 2006; Wahjudi et al. 2011; Hong et al. 2012). Cleavage and therewith inactivation of C12-HSL leaves its cognate regulator protein LasR vacant and thus inactive, resulting in decreased downstream activation of the regulatory systems. However, none of the three abovementioned acylases are able to degrade the short chain autoinducer C4-HSL (Waters and Bassler 2005; Wahjudi et al. 2011). So far, no acylase has been described in *P. aeruginosa*, which is able to cleave C4-HSL. The inactivation of autoinducer signalling leads to vacant and hence dysfunctional RhlR, lacking its corresponding C4-HSL autoinducer molecule. Orphan regulator RhlR was previously shown to act as a repressor of *rhlAB* transcription (Medina et al. 2003c). During the fermentation process, once the culture reached lysis phase, *rhlR* expression maintained the same high level it had reached during the late deceleration phase. All other components of the *rhl*, *las*, and *pqs* system were downregulated in their relative expression.

Furthermore, C4-HSL levels were monitored during the fermentation process and confirmed the assumption of autoinducer degradation, as C4-HSL concentrations increased until mid-deceleration phase and thereafter decreased. Briefly, preliminary experiments revealed an acylase-mediated C4-HSL degradation during the presented bioprocess that can be clearly linked to the culture supernatant rather than the cell fraction, implicating an exo-enzymatic mechanism for C4-HSL degradation (Henkel et al. 2013). A concurrent decline in *rhlI* expression that has been observed during the end of the batch fermentation could lead to a lack of *de novo* synthesis of C4-HSL, further causing a shift in the C4-HSL-RhlR-balance. Consequently, RhlR might be orphaned by the time the culture reached lysis phase due to increased *rhlR* expression. However, it has been reported that neither overexpression of *rhlR* nor exogenous addition of C4-HSL activated *rhlA* expression (Medina et al. 2003b; Schuster and Greenberg 2007). It remains an open question, if C4-HSL limitation plays a major role in rhamnolipid production as it is subjected to a multilayered regulatory network. Quantification of protein levels of both RhlR and RhlR-C4-HSL complex could give critical indication on repressive versus activating properties of orphan and complex-bound RhlR during the time course of the fermentation process. Although both *rhlA* and *rhlC* expression remained detectable until the end of the fermentation process, the rhamnolipid productivity reached its maximum much earlier, i.e., after 22 h, suggesting that additional metabolic effects might be responsible for the regulation of rhamnolipid production at this later stage of the process.

The *rpoS* gene has been shown to be expressed during stationary growth phase and is required for the expression of numerous genes in response to various stresses, such as nutrient limitation, osmotic pressure, and growth during the stationary phase (Bertani et al. 2003). Monitoring *rpoS* expression may also constitute a potent marker for good culture viability and metabolite producing capability. In the presented fermentation process, the gene for the stationary sigma factor, *rpoS*, was seen to be upregulated until lysis phase and was then downregulated at the beginning of the lysis phase after 40 h. Downregulation of *rpoS* could be resultant of a loss of viability and lysis of the culture. However, as metabolic shortage or nutrient starvation, such as nitrogen or iron limitation functions as a trigger for rhamnolipid synthesis rather than preventing its production, it appears unlikely that the decrement in rhamnolipid productivity rate after 22 h is due to metabolic reasons. Furthermore, during cell lysis critical components might be released from dying cells that may stimulate expression of rhamnolipid synthesis genes at this late stage of fermentation. This might help explain, why expression of both *rhlA* and rhlC remains at a measurable activity and is not shut down completely during lysis phase.

All in all, a gene regulatory stock has been taken and further steps towards selective and targeted process control based upon the established network can be done. For proof of principle, we could show that nitrogen limitation caused a more than 20-fold upregulation of the genes for rhamnolipid synthesis compared to standard medium conditions. This

demonstrates the potential of this monitoring system to function as a useful tool for identifying potential new set-screws, with which the process can be positively manipulated. For instance, the use of specific acylase or QS inhibitors could be helpful to maintain rhamnolipid production during batch fermentation, which can easily be controlled by such gene expression analyses of switchpoints.

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iv. Influence of Ferric Iron on Gene Expression and Rhamnolipid Synthesis during Batch Cultivation of *Pseudomonas aeruginosa* PAO1

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Influence of ferric iron on gene expression and rhamnolipid synthesis during batch cultivation of *Pseudomonas aeruginosa* PAO1

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Abstract Bioprocesses based on sustainable resources and rhamnolipids in particular have become increasingly attractive in recent years. These surface-active glycolipids with various chemical and biological properties have diverse biotechnological applications and are naturally produced by *Pseudomonas aeruginosa*. Their production, however, is tightly governed by a complex growth-dependent regulatory network, one of the major obstacles in the way to upscale production. *P. aeruginosa* PAO1 was grown in shake flask cultures using varying concentrations of ferric iron. Gene expression was assessed using quantitative PCR. A strong increase in relative expression of the genes for rhamnolipid synthesis, *rhlA* and *rhlC*, as well as the genes of the *pqs* quorum sensing regulon was observed under iron-limiting conditions. Iron repletion on the other hand caused a down-regulation of those genes. Furthermore, gene expression of different iron regulation-related factors, i.e. *pvdS*, *fur* and *bqsS*, was increased in response to iron limitation. Ensuing from these results, a batch cultivation using production medium without any addition of iron was conducted. Both biomass formation and specific growth rates were not impaired compared to normal cultivation conditions. Expression of *rhlA*, *rhlC* and *pvdS*, as well as the gene for the 3-oxo-C12-HSL synthetase, *lasI*, increased until late stationary growth phase. After this time point, their expression steadily decreased. Expression of the C4-HSL synthetase gene, *rhlI*, on the other hand, was found to be highly increased during the entire process.

Keywords *Pseudomonas aeruginosa* PAO1 · Rhamnolipid · Biosurfactant · Gene expression · Iron starvation · Quorum sensing

Introduction

Rhamnolipids are surface-active glycolipids, which have gained major attention in the recent years as they represent a promising alternative to petrochemical surface-active components. Amongst microbial biosurfactants, rhamnolipids are one of most eagerly studied (Henkel et al. 2012). However, rhamnolipid production remains a characteristic for *Pseudomonas aeruginosa* (Icks et al. 1997), and the highest yield so far has been achieved using this Gram-negative, opportunistic, human pathogen (Patent number US5501966). So far, industrial production has not yet been achieved in a large scale, which is due to various drawbacks such as low product yields, excess foaming, relatively costly raw materials and expensive downstream processing (Banat et al. 2000). Additionally, the complex regulatory network behind rhamnolipid synthesis in *P. aeruginosa* constitutes a major obstacle in the way to upscale production. This makes it even more crucial to fully understand the regulatory mechanisms behind rhamnolipid synthesis.

P. aeruginosa is known to produce four types of rhamnolipid species, the most common however are RL1

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($RhaC_{10}C_{10}$) and RL3 ($Rha_2C_{10}C_{10}$), designated mono- and dirhamnolipids, respectively (Rahman et al. 2002). Synthesis of RL precursors, namely 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA), is catalysed by the rhamnosyltransferase 1 subunit A, RhlA (Deziel et al. 2003). Those HAA moieties are subsequently transferred onto a dTDP-L-rhamnose unit by the catalytic protein of the transferase, the rhamnosyltransferase 1 subunit B (RhlB) to form monorhamnolipids (Deziel et al. 2003; Soberon-Chavez et al. 2005). Subunits A and B are encoded in one mutual operon and were shown to be quorum sensing (QS)-regulated via the QS regulator protein RhlR (Deziel et al. 2003; Medina et al. 2003c; Soberon-Chavez et al. 2005; Upritchard et al. 2007). Synthesis of dirhamnolipids is then catalysed by the rhamnosyltransferase 2 (RhlC) from monorhamnolipids (Rahim et al. 2001). However, there is much more to rhamnolipid regulation than cell density and additional layers of regulation have been described (Schuster and Greenberg 2006). For one, *rhlAB* expression is partially dependent on the alternative sigma factor RpoS (Medina et al. 2003b). Other studies link rhamnolipid synthesis to nutritional conditions, such as nitrogen exhaustion, which is mediated by the alternative sigma factor for nitrogen limitation, RpoN (Rahim et al. 2001; Medina et al. 2003a, c). Several other QS-related and QS-unrelated transcriptional as well as posttranscriptional regulation mechanisms that influence rhamnolipid synthesis have been described (Müller and Hausmann 2011).

Another interesting stimulus for rhamnolipid synthesis is iron limitation, which constitutes an essential trace element for many species, and iron availability is of major importance not only for *P. aeruginosa*, but for many other bacteria as well (Cornelis 2010; Glick et al. 2010). The competition for iron between pathogens and its host is even critical for the outcome of a disease (Skaar 2010). Under aerobic conditions, which pseudomonads as facultative aerobic microorganisms are most likely to encounter naturally, iron occurs in its oxidised form, Fe^{3+}, ferric iron (Vasil and Ochsner 1999). Unlike the soluble ferrous iron Fe^{2+}, Fe^{3+} is poorly soluble under aerobic conditions (Braun and Killmann 1999). As iron is scarce in natural surroundings, such as soil or eukaryotic hosts, several strategies for the uptake of iron have been developed by *P. aeruginosa* including the production of high-affinity iron-binding compounds, so-called siderophores (Cornelis 2010). However, host protein-bound iron can also be utilised by direct uptake or enzymatic degradation of those compounds (Cornelissen and Sparling 1994; Wolz et al. 1994). Additionally, ferric iron can be reduced to the soluble form, ferrous iron (Coulanges et al. 1997). Iron uptake requires tight regulation, since both forms are highly reactive and are able to form potentially harmful reactive oxygen species (Andrews et al. 2003). In turn, many genes of *P. aeruginosa* including those for iron uptake or several virulence factors are subjected to iron-dependent regulation, which is mainly mediated by the regulator protein Fur (Ferric uptake regulator) and a Fur-regulated alternative sigma factor PvdS (Vasil and Ochsner 1999; Frangipani et al. 2013). Interestingly, some of the iron-regulated genes were found to be QS-regulated as well (Cornelis and Aendekerk 2004). In *P. aeruginosa*, iron starvation has been described to be mediated by a two-component system and mutations resulted in a massive reduction in rhamnolipid synthesis, as well as reduced C4-HSL and PQS signal production (Upritchard et al. 2007; Kreamer et al. 2012). Iron is furthermore one of the major turnstiles, when it comes to physiology, and its availability is responsible for crucial changes in lifestyle and growth behaviour (Berlutti et al. 2005; Musk et al. 2005; Yang et al. 2007). Transition from the free-living to biofilm mode of growth has been ascribed to both iron and rhamnolipids (Berlutti et al. 2005; Patriquin et al. 2008; Glick et al. 2010). During early biofilm development, rhamnolipid production has been demonstrated to be increased under iron-limiting conditions, which in turn induced twitching motility (Patriquin et al. 2008). Rhamnolipid production was also shown to be shifted to initial stages of biofilm development under iron limitation rather than delayed under iron-replete conditions (Glick et al. 2010). Facilitated surface motility and consequent detachment might enable the bacterium to access more favourable surroundings, if the current conditions are inconvenient.

In a previous study (Schmidberger et al. 2013), a molecular biology-based strategy for understanding the regulation of rhamnolipid synthesis during batch cultivation was established. Briefly, this study focused on generating expression profiles of different factors that have previously been described to be involved in the regulation of rhamnolipid synthesis. The method for generating the expression profiles was quantitative PCR, which allows the assessment of gene expression at the most dynamic level. Using this monitoring system might help in identifying potential new possibilities, with which the process can be positively manipulated. In the current study, the same *P. aeruginosa* PAO1 DSM22644 strain was confronted with various concentrations of ferric iron to evaluate if iron limitation can be used to evoke an increased rhamnolipid production. For this purpose, the previously established monitoring system was used. Hence, the expression of the genes for rhamnolipid synthesis, as well as the genes of the regulatory circuitry, was under investigation. To begin with, preliminary experiments were conducted in shake flasks. Based upon these small-scale experiments, a batch cultivation process under iron-limiting conditions was conducted with the same strain to investigate the potential for biotechnological upscale production. Gene expression of the same factors was monitored over the entire time course of the cultivation to receive an insight over the regulatory network.

Materials and methods

Bacterial strains

Pseudomonas aeruginosa PAO1 (DSM 22644) was used for shake flask experiments, as well as for batch cultivation experiments. For gfp expression analyses, *P. aeruginosa* wild-type lab strain H103, as well as *Escherichia coli* DH5α was used.

Shake flask cultivations

Planktonic cultures were grown in BM2 minimal medium for approximately 6 and 24 h at 37 °C in a rotary shaker (MaxQ 4000, Thermo Scientific) at 170 rpm in shake flasks allowing optimal aeration. BM2 minimal medium for cultivation was prepared as previously described (Schmidberger et al. 2013). Iron starvation was achieved by omitting Fe^{3+} in the BM2 medium, while iron repletion was achieved by using the 10-fold amount of Fe^{3+} compared to standard BM2, where iron is 10 μM. Iron titration was done by varying the amount of Fe^{3+} in the medium ranging from 1, 2, 5, 10, 20 to 100 μM.

Preparation of seed cultures for batch cultivation

Pre-cultures of *P. aeruginosa* PAO1 were prepared from glycerol stocks using a total volume of 100 μL stock solution in 25 mL lysogeny broth (LB). Overnight cultivation was conducted at 37 °C in 250 mL baffled shake flasks at 120 rpm (Multitron II, HT Infors, Bottmingen, Switzerland). Prior to batch cultivation, 5 mL of the pre-culture was inoculated in 200 mL production medium supplemented with 25 g ($\delta_{sunflower\ oil}$=0.921 g/mL) sunflower oil at 37 °C in 1 L baffled shake flasks at 120 rpm. After 18 to 20 h of incubation, an appropriate volume of the culture was used to inoculate the bioreactor. For this purpose, a theoretical OD_{580} of 0.05 was used.

Iron limited batch cultivation of *P. aeruginosa* PAO1 in a 2.2-L bioreactor

Batch cultivations of *P. aeruginosa* PAO1 DSM22644 were carried out in a benchtop bioreactor (MiniFors, Infors HT, Bottmingen, Switzerland) equipped with a 2.2-L glass vessel and a Rushton turbine as a stirring device. Cultivations were performed with a total liquid volume of 800 mL in production medium and sunflower oil, as described previously (Müller et al. 2010), omitting iron in the initial addition of trace elements and all subsequent supplementations. Briefly, this production medium was composed of a Ca-free mineral salt solution with 15.0 g/L $NaNO_3$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L KCl and 0.3 g/L K_2HPO_4. As sole carbon source, sunflower oil with a starting concentration of 250 g/L was used and 1 mL/L of trace element solution was added. The trace element solution contained 2.0 g/L sodium citrate·$2H_2O$, 1.4 g/L $ZnSO_4 \cdot 7H_2O$, 1.2 g/L $CoCl_2 \cdot 6H_2O$, 1.2 g/L $CuSO_4 \cdot 5H_2O$ and 0.8 g/L $MnSO_4 \cdot H_2O$. Mineral salt solutions, phosphate sources and sunflower oil were autoclaved separately for all experiments. Trace elements were filter-sterilised through a 0.22-μm membrane filter (Carl Roth GmbH, Karlsruhe, Germany). Production medium was adjusted to pH 6.5 and controlled during cultivation using 4 M NaOH or 4 M H_3PO_4, respectively. To account for foaming during cultivation, a 5-L stirred foam trap containing 4 M H_3PO_4 was connected to the exhaust gas cooler. To minimise iron contamination of the media, shrink tubing was applied to cover metallic surfaces of the stirring shaft, baffles and aeration tube. pH was maintained at 6.50 with 4 M NaOH and 4 M H_3PO_4, and the pO_2 was controlled by varying stirrer speed from 500 to 1,200 rpm at a constant aeration rate of 0.2 L/(L min).

Sampling

Samples from shake flask experiments were taken 6 and 24 h after inoculation and treated with RNAProtect® Bacteria Reagent from QIAGEN (Hilden, Germany) for RNA stabilisation according to the manufacturer's instructions. Samples were then stored at −80 °C until sample preparation. Optical densities were measured spectrometrically at 600 nm wavelength prior to sampling using a GENESYS 10S UV-vis spectrophotometer (Thermo Scientific, Frankfurt, Germany). Samples for offline molecular analysis of the cultivation process were taken approximately every 6 to 8 h, starting at about 16 h post-inoculation in case of sufficient biomass accumulation. Calculations for gene expression ratios are based upon this time point. RNA stabilisation and storage was conducted as described above. Furthermore, samples for offline analysis of bio dry mass (BDM), as well as mono-/dirhamnolipid concentration, were routinely taken.

RNA isolation and reverse transcription

Total RNA was isolated after RNA stabilisation by phenol/chloroform extraction using QIAGEN's QIAzol® Lysis Reagent in combination with the RNeasy® Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was subsequently digested using TURBO® DNase (Invitrogen, Darmstadt, Germany), and successful digestion was then evaluated by using polymerase chain reaction followed by loading eventual products on an agarose gel. To control RNA integrity as well as quality, 260/280 and 260/230 ratios were assessed photospectrometrically (NanoDrop® ND-1000, Thermo Scientific, Frankfurt, Germany). RNA concentrations were then measured and a total of 250 ng RNA was used for complementary DNA (cDNA) synthesis. For each sample, an equal amount of RNA was transcribed into cDNA.

Random-primed reverse transcription was conducted using MultiScribe® Taqman Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. The temperature profile was according to protocol (25 °C, 10 min; 48 °C, 30 min; 95 °C, 5 min; 4 °C, ∞). After reverse transcription, samples were directly used for real-time PCR or otherwise stored at −20 °C.

Gene expression analysis using quantitative real-time polymerase chain reaction (RT-PCR)

Gene expression was assessed using a SYBR Green mediated two-step quantitative real-time PCR. Prior to PCR, RNA was first transcribed into cDNA as described above, and in a second step, the quantitative real-time PCR was performed using an ABI PRISM® 7300 (Applied Biosystems, Darmstadt, Germany) in combination with KAPA® SYBR FAST qPCR ABI PRISM (Peqlab, Erlangen, Germany). Primers used for real-time PCR are listed in Table 1. Prior to gene expression analyses, primers were checked for appropriate product amplification, and primer efficiencies were determined and target specific efficiencies were then used for calculation of relative expression ratios. All primers used met the requirements as well as the tolerable efficiency range of 90 to 110 % or were redesigned if otherwise.

Molecular data analysis

Gene expression data was obtained using the ABI PRISM® 7300 associated SDS Software (v1.4, Applied Biosystems, Darmstadt, Germany) and subsequently analysed using the Qbase Plus software from BioGazelle (Zwijnaarde, Belgium), which calculates relative gene expression based on the delta-delta Ct method (Pfaffl 2001; Hellemans et al. 2007). As reference genes for relative quantification, calculations served the housekeeping genes, *rpoD*, the major sigma (σ^{70}), and *fabD*, the malonyl-CoA-[acyl-carrier-protein]-transacylase, which have previously been shown to be stably expressed in *P. aeruginosa* and are hence considered suitable as reference genes (Savli et al. 2003; Potvin et al. 2008). Regarding the expression profiles, equal amounts of cDNA were used for each sample. The expression of a particular target gene at a certain time point during the cultivation was compared with the expression of the same target gene at the start of the process that is after 16 h of cultivation. Therefore, the expression of all target genes of interest at 16 h is considered to be one. This expression was then normalised against the two reference genes. For shake flask experiments, gene expression of target genes in standard BM2 medium served as a reference.

RhlA_gfp and *rhlC_gfp* expression analyses

The *rhlA_gfp* fusion vector has been previously described (Lequette and Greenberg 2005). Briefly, the *rhlA* promoter region was amplified from chromosomal *P. aeruginosa* PAO1. The amplified product extends 551 bp upstream of the *rhlA* translational start and was cloned in front of the promoterless gfp in pPROBE-AT vector. This *rhlA_gfp* fusion vector, transformed into *Escherichia coli* DH5α, was kindly provided by Professor Greenberg from the University of Washington, USA. Subsequently, the *rhlA_gfp* fusion vector was isolated using the GeneJet PCR Purification Kit (Fermentas, Thermo Scientific, Frankfurt, Germany), checked for the presence of insert and vector by digesting it with *Eco*RI and *Hin*dIII and introduced into competent *P. aeruginosa* PAO1 H103 wild type strain for gfp measurement experiments. The *rhlC_gfp* fusion strain was constructed as follows. The *rhlC* promoter region was amplified from *P. aeruginosa* PAO1 H103 chromosomal DNA by PCR with 5′-aaaaaaagcttAGAGGCTGGTCGTGGACA-3′ and 5′-aaaaaggtaccACGGCAGGTAGCTTTCCTTT-3′ using the KAPA HiFi Fidelity polymerase from (Peqlab, Erlangen, Germany). The cloned fragment is 273 bp and extends from 254 bp upstream of the *rhlC* translational start containing the AGGAGG-Shine-Dalgarno sequence for ribosome binding. The amplified product was then isolated from an agarose gel using the GeneJET PCR Gel Extraction Kit (Fermentas, Thermo Scientific, Frankfurt, Germany) according to the manufacturer's instructions. The product was then double-digested with *Kpn*I and *Hin*dIII (Fermentas, Thermo Scientific, Frankfurt, Germany) using Kpn buffer. The gfp containing vector, pPROBE-AT′, was digested with *Kpn*I and *Hin*dIII separately from the insert under same reaction conditions. Successful digestion was controlled by an agarose gel after purifying the fragments using the GeneJet PCR Purification Kit (Fermentas, Thermo Scientific, Frankfurt, Germany). Vector and insert were subsequently ligated using T4 DNA Ligase (Fermentas, Thermo Scientific, Frankfurt, Germany) to generate the *rhlC_gfp* fusion vector. Subsequently, transformation of the *rhlC_gfp* fusion vector into competent *E. coli* DH5α was done by heat transformation. Positive selection was conducted on LB agar containing ampicillin (100 mg/mL). The *rhlC_gfp* fusion vector was consequently isolated using the GeneJET Plasmid Miniprep KIT (Fermentas, Thermo Scientific, Frankfurt, Germany) and introduced into competent *P. aeruginosa* PAO1 H103 wild-type strain using electroporation. Positive clones were subsequently selected using LB agar containing carbenicillin (300 mg/mL). For the gfp, as well as the growth measurements, an Infinite F200 Pro microplate reader and associated software (Tecan Group Ltd.) were used. The emission wavelengths were 485 and 535 nm for gfp fluorescence and 600 nm for absorbance measurements, respectively. The measured gfp

Table 1 Primers for quantitative real-time PCR

Primer or target	Sequence	Source or reference	Amplicon size (bp)
lgR	F 5′-ATCAGTGCACGGACCCGCAA-3′ R 5′-GCATGGCGCAAGGTCACGTA-3′	Schmidberger et al. (2013)	95
bqsR	F 5′-CAGTGCGTGTGCCTGAAC-3′ R 5′-GCGGGTCTCGATCACCT-3′	Kreamer et al. (2012)	212
bqsS	F 5′-CTGGCGCACATTTCGATT-3′ R 5′-ACTTCGCTATCGGCCCACTT-3′	Kreamer et al. (2012)	190
fabD	F 5′-GCATCCCTCGCATTCGTCT-3′ R 5′-GGCGCTCTTCAGGACCATT-3′	Savli et al. (2003)	163
fur	F 5′-GTGATCGAGTTCATGGATGC-3′ R 5′-GCACGTAGAGCACCAGATTG-3′	This study	100
gacA	F 5′-ATATCAGCCCGCAGATCGCCCA-3′ R 5′-TTCGGCGACAGGCACAGCTT-3′	Schmidberger et al. (2013)	166
lasI	F 5′-GCCCCTACATGCTGAAGAACA-3′ R 5′-CGAGCAAGGCGCTTCCT-3′	Schwartz et al. (2007)	62
lasR	F 5′-GTGGAGCGCCATCCTGCAGA-3′ R 5′-CGGTCGTAATGCTCGCGCCA-3′	Schmidberger et al. (2013)	144
lipC	F 5′-ACTACACCCGCACGCGCTAT-3′ R 5′-GTTGCTGTTCACCGGCGACT-3′	Schmidberger et al. (2013)	155
mvfR	F 5′-ATCAAGCAGGACAACGCGGA-3′ R 5′-GCAGGGAGGCATTGCACAAC-3′	Lee et al. (2011)	165
pqsE	F 5′-GCGCTTGAACCGGCAACTGT-3′ R 5′-GGCGTCGCACGTCGTAGAAA-3′	Schmidberger et al. (2013)	185
pvdS	F 5′-AGATGTGGTCCAGGATGCGT-3′ R 5′-GTGTTCGAGGGTCGCGTAGT-3′	Viducic et al. (2007)	232
qscR	F 5′-AAGCAATGTGCGCCTGACCG-3′ R 5′-TTCACCGTGCGCTGGTCGAT-3′	Schmidberger et al. (2013)	111
rhlA	F 5′-GATCGAGCTGGACGACAAGTC-3′ R 5′-GCTGATGGTTGCTGGCTTTC-3′	Schmidberger et al. (2013)	95
rhlC	F 5′-ATCCATCTCGACGGACTGAC-3′ R 5′-GTCCACGTGGTCGATGAAC-3′	Neilson et al. (2010)	159
rhlI	F 5′-AGCTGGGACGCTACCGGCAT-3′ R 5′-TGGCGGCTCATGGCGACGAT-3′	Schmidberger et al. (2013)	136
rhlR	F 5′-GAGGAATGACGGAGGCTTTTTG-3′ R 5′-CTTCTTCTGGATGTTCTTGTGG-3′	Kayama et al. (2009)	255
rpoD	F 5′-GGGCGAAGAAGGAAATGGTC-3′ R 5′-CAGGTGGCGTAGGTGGAGAA-3′	Savli et al. (2003)	178
rpoN	F 5′-ACCCGTAGTAGTGGATGGTGC-3′ R 5′-TATGGCCTGTTGCAGCTGCG-3′	Bazire et al. (2005)	128
rpoS	F 5′-CTCCCCGGGCAACTCCAAAAG-3′ R 5′-CGATCATCCGCTTCCGACCAG-3′	Savli et al. (2003)	198
rsaL	F 5′-AGCCCCAAAACATGGCCTTCCG-3′ R 5′-CTCGAAACGACTGCCGCAGGAT-3′	Lee et al. (2011)	113
rsmA	F 5′-GAGACCCTGATGGTAGGTGACGACG-3′ R 5′-CGATGCGCACCTGGTTCCCTTT-3′	This study	70

fluorescence emission was then correlated to the measured optical density of each culture to produce a specific gfp fluorescence.

Rhamnolipid quantification using liquid chromatography tandem mass spectrometry (LC/MS/MS)

Mono- and dirhamnolipids were quantified in the cell-free culture supernatant of the shake flask experiments using liquid chromatography tandem mass spectrometry (LC/MS/MS). For this purpose, three volumes of culture supernatants were treated with five volumes of chloroform to extract rhamnolipids, which are consequently located in the organic phase. After freezing the samples and subsequent phase separation, the organic phase was isolated and chloroform evaporated using a heating block and a stream of gaseous N_2. The remaining dry substance was then resuspended in CH_3CN/10 mM NH_4OAc (50:50), and the samples were analysed using an API 365 LC/MS/MS System (Perkin Elmer). Purified mono- and dirhamnolipid served as a reference for calibration. The rhamnolipid quantification was conducted by Michael Nusser (Karlsruhe Institute of Technology, Institute for Functional Interfaces).

Results

To investigate the influence of iron starvation on the regulatory circuitry around rhamnolipid synthesis, experiments in shake flasks were conducted, first. *P. aeruginosa* PAO1 (DSM22644) was cultivated under different deplete and replete conditions of Fe^{3+}. To capture both early and late growth phase responses, samples were taken during mid-exponential phase after approximately 6 h of incubation and during stationary phase of growth after 24 h. Media with concentrations of ferric iron ranging from 0, 1, 2, 5, 10 to 100 μM were used for cultivation. Standard BM2 medium served as reference for the calculation of gene expression ratios. Common laboratory use, including that in BM2 medium, is 10 μM iron, whereas concentrations between up to 100 μM are considered as replete conditions for *P. aeruginosa* (Vasil and Ochsner 1999). BM2 medium without the addition of iron served as iron starvation medium. Concentrations that ranged between 0 and 10 μM Fe^{3+} were considered as iron-limited conditions.

In addition to evaluating gene expression, optical density (OD_{600}) was measured directly before sampling to figure out possible growth impairments caused by iron starvation or repletion, which might be detrimental for biotechnological upscale production. Consequently, overnight growth curves for *P. aeruginosa* PAO1 under different iron concentrations were made. Until mid-exponential phase of growth, iron limitation did not cause a significant impairment. However, the optical density was slightly lower in cultures grown with 0, 1 and 2 μM Fe^{3+} (Fig. 1). Those cultures reached an OD_{600} of approximately 0.5, whereas cultures above 2 μM, i.e. 5, 10 and 100 μM, reached an OD_{600} of more than 0.6. The

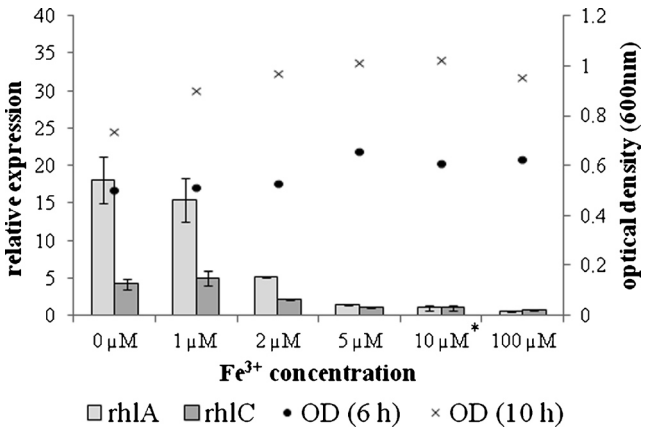

Fig. 1 Relative gene expression of rhamnolipid synthesis genes (*rhlA* and *rhlC*, respectively) in *P. aeruginosa* PAO1 in response to various concentrations of Fe^{3+}. The gene expression of *rhlA* and *rhlC* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at different concentrations of Fe^{3+} during shake flask experiments. Samples were collected at mid-exponential phase after 6 h of cultivations. Expression of genes in BM2 medium with 10 μM Fe^{3+} was used as a reference for the calculation of expression ratios and is indicated by the *asterisk*. The optical density (600 nm) was determined after 6 and 10 h of cultivation

difference in growth was more prominent after cultures reached stationary growth phase. While cultures grown under iron-limiting conditions had an OD_{600} of 0.74 after 10 h of cultivation, cultures grown under standard conditions had an OD_{600} of 1.024 (Fig. 1).

Expression of both *rhlA* and *rlhC* was increased under iron-starving conditions compared to standard BM2 medium that is more than 18-fold for *rhlA* and 4-fold for *rhlC*, respectively (Fig. 1). In the case of iron limitation (2 μM Fe^{3+}), expression of *rhlA* was increased by 5-fold, whereas expression of *rhlC* was increased by 5-fold. Iron reduction (5 μM Fe^{3+}) did not have an effect on their expression. Iron repletion with 100 μM Fe^{3+} on the other hand dampened the expression, and both *rhlA* and *rhlC* expressions were reduced to expression levels under one compared to standard conditions.

As rhamnolipid synthesis is also quorum sensing-regulated, the effect of iron starvation on quorum sensing was investigated. Therefore, central components of the three QS systems of *P. aeruginosa*, i.e. the *las*, *rhl* and *pqs* systems, were investigated. The most prominent effect could be seen for the *pqs* system. Both the *mvfR* (*pqsR*) as well as the *pqsE* were strongly up-regulated under iron-limiting conditions (Fig. 2a). Whereas *pqsE* was only slightly up-regulated under iron depletion, *mvfR* showed enhanced expression of more than 7-fold compared to the reference. The *rhl* system responded to a much lesser extent. The gene for the synthetase, *rhlI*, was up-regulated 2- and 4-fold under iron starvation and limitation, respectively (Fig. 2b). The *rhlR* gene, coding for the corresponding regulator protein, was up-regulated in concentrations from 0 to 2 μM, whereas its expression above this concentration was as under standard conditions. The *las* system did not show a dependency to different iron conditions and the expression of both *lasR* and *lasI* was not up-regulated in response to different iron concentrations (data not shown). Common for all regulator genes is their low expression under iron repletion compared to the reference.

Furthermore, the influence of ferric iron on the expression of several sigma factors was investigated. The expression of the gene for the sigma factor for nitrogen limitation, *rpoN*, was not strongly affected by varying iron concentrations, and its expression remained low even under iron-limiting conditions (Fig. 3a). The gene for the stationary phase sigma factor, *rpoS*, on the other hand was up-regulated in the case of no or reduced iron concentrations (0–2 μM) (Fig. 3a). However, under more replete conditions of 100 μM Fe^{3+}, *rpoS* was also slightly up-regulated compared to standard conditions. For the sigma factor for iron starvation, *pvdS*, an up-regulation of almost 5-fold could be observed under iron-limiting conditions from 0 to 2 μM Fe^{3+} (Fig. 3b). Under iron-replete conditions, its expression was down-regulated to 0.1-fold.

Additionally, various factors that are involved in iron-dependent regulation or uptake were investigated. The two-component system BqsS/BqsR that senses extracellular iron

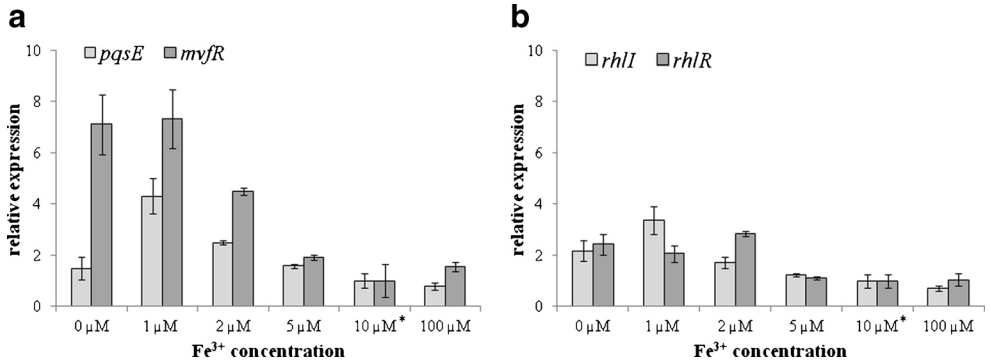

Fig. 2 Relative expression of the genes of the *pqs* and *rhl* QS systems in *Pseudomonas aeruginosa* PAO1 in response to various concentrations of Fe^{3+}. Gene expression of the *pqs* QS components is shown on the *left* (**a**), while the *rhl* QS system is depicted on the *right* (**b**). The expression of *pqsE*, *mvfR* (*pqsR*), *rhlI* and *rhlR* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at different concentrations of Fe^{3+} during shake flask experiments. Samples were collected at mid-exponential phase after 6 h of cultivations. Expression of genes in BM2 medium with 10 µM Fe^{3+} was used as a reference for the calculation of expression ratios and is indicated by the *asterisk*

showed a strong up-regulation of almost 12-fold of the gene for the signal response protein, *bqsS*, under iron starvation and 4-fold under iron limitation (Fig. 4a). The gene for the corresponding regulator protein, *bqsR*, did not show any response in dependency on iron availability and its expression did not vary compared to standard BM2 concentrations of Fe^{3+}. The expression of the gene for the ferric uptake regulator, *fur*, was shown to be up-regulated 6-fold under iron-limiting conditions of 1 µM Fe^{3+}, whereas under iron omission, *fur* was up-regulated only by approximately 3-fold (Fig. 4b). Under iron limitation, the gene for the QS regulator protein, *rsaL*, was up-regulated 4-fold. With 1 µM Fe^{3+}, *rsaL* was also up-regulated, however only by 2-fold, whereas the gene for the quorum sensing control repressor, *qscR*, was up-regulated 4-fold (Fig. 4c). Additionally, the gene expression of different other QS-associated regulatory factors was assessed, including *gacA*, *vfR*, *lipA* and *qslA*. However, there was no response to varying iron concentrations and the data is hence not shown.

To verify the results obtained by gene expression analyses, rhamnolipid production was assessed in the culture supernatant using LC/MS/MS. Briefly, cultures grown under iron limitation showed increased amounts of extracellular rhamnolipid compared to cultures grown in standard BM2 or under iron-replete conditions (Fig. S1). Noteworthy is that under iron limitation, production of dirhamnolipids was favoured.

In order to examine the timing of rhamnolipid synthesis gene expression under different iron concentrations, gfp assays were conducted using two gfp fusion vectors, namely a *gfp_rhlA* and a *gfp_rhlC* fusion vector. Specific *rhlA_gfp* expression was not only increased under iron-limiting conditions, but started to increase much earlier compared to higher concentrations of Fe^{3+} (Fig. 5a, b). In cultures lacking supplemented iron, *rhlA_gfp* expression started to increase approximately 1 h earlier than in cultures grown in standard BM2 medium (Fig. 5a). *RhlC_gfp* expression on the other hand did

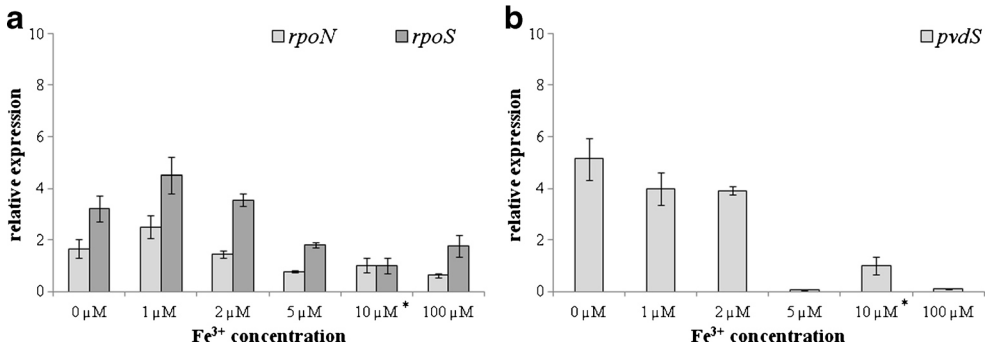

Fig. 3 Relative gene expression of sigma factors RpoN, RpoS and PvdS in *P. aeruginosa* PAO1 in response to various concentrations of Fe^{3+}. Sigma factors for nitrogen limitation and stationary phase growth are depicted on the *left* (**a**), while the sigma factor for iron limitation is shown on the *right* (**b**). The gene expression of *rpoN*, *rpoS* and *pvdS* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at different concentrations of Fe^{3+} during shake flask experiments. Samples were collected at mid-exponential phase after 6 h of cultivations. Expression of genes in BM2 medium with 10 µM Fe^{3+} was used as a reference for the calculation of expression ratios and is indicated by the *asterisk*

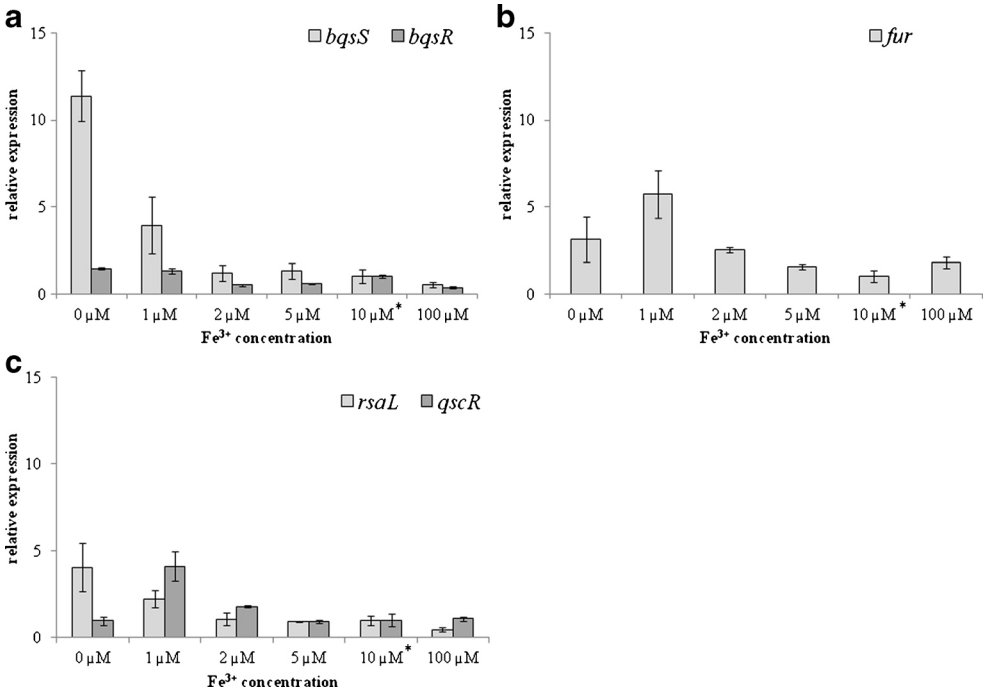

Fig. 4 Relative gene expression of regulatory factors involved in iron uptake in *P. aeruginosa* PAO1 in response to various concentrations of Fe^{3+}. The BqsS/BqsR two-component system is depicted on the *upper left panel* (**a**), while the ferric uptake regulator, *fur*, is shown on the *upper right panel* (**b**) and the regulatory factors, *rsaL* and *qscR*, in the *lower left panel* (**c**). The gene expression of *bqsS*, *bqsR*, *fur*, *rsaL* and *qscR* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at different concentrations of Fe^{3+} during shake flask experiments. Samples were collected at mid-exponential phase after 6 h of cultivations. Expression of genes in BM2 medium with 10 µM Fe^{3+} was used as a reference for the calculation of expression ratios and is indicated by the *asterisk*

not appear much earlier under iron-limiting compared to standard conditions (Fig. 5b). During exponential growth phase, varying iron concentrations seemed to have a stronger effect on *rhlA_gfp* expression than *rhlC_gfp*. By the time cultures had reached stationary phase, effects of varying iron concentrations became more apparent. Both *rhlA_gfp* and *rhlC_gfp* expressions act reciprocal to iron availability and decreased with increasing iron concentrations. Under iron depletion, expression of *rhlA_gfp* and *rhlC_gfp* was highest.

As iron limitation clearly had an effect on rhamnolipid synthesis, *P. aeruginosa* PAO1 was grown with iron-free production medium using sunflower oil as sole carbon source in a small batch cultivation process using a MiniFors 2.2-L bioreactor. Both biomass formation and concentrations of mono- and dirhamnolipids showed logistic behaviour. The maximal specific growth rate (µmax) was reached after approximately 25 h of cultivation. The specific rhamnolipid production rate (qRL) was at a maximum after approximately 44 h, which corresponds with late stationary phase of growth (Fig. 6). Both bio dry mass formation and specific growth rates were comparable to the standard batch process using a Biostat® Cplus 42-L bioreactor (Schmidberger et al. 2013) and were not impaired in response to the iron limitation.

To investigate the regulatory activity of the genes of interest, the distribution of their Ct values over the entire time course of the process was assessed for the larger scaled 42-L batch cultivation under standard conditions (Schmidberger et al. 2013). Likewise, the distribution of Ct values was analysed in a boxplot diagram for the current 2.2-L batch process under iron-free conditions as well (Fig. S2). Briefly, Ct values of the target genes, as well as the housekeeping genes, were generally lower for the current process than they were for the standard batch. However, the transcription range of each gene in comparison to the other genes was comparable to the standard batch, so that no gene was exceptionally more regulated in the iron-limited process.

Gene expression analyses of the current batch cultivation under iron-free conditions showed that *rhlA* started to slightly increase 30 h into cultivation by almost 10-fold (Fig. 7). However, at 55 h of cultivation, *rhlA* expression was strongly up-regulated and reached a maximum expression of more than 30-fold compared to the reference point at 16 h. Over the rest of the process, *rhlA* expression decreased steadily until it reached a relative expression of more than 10-fold by the end of the process. *RhlC* expression on the other hand did not show any significant up-regulation until 55 h of cultivation, where it was up-regulated by more than 3-fold (Fig. 7). Its expression then reached another peak after 72 h of cultivations, where it was expressed 4-fold higher than at the reference time point. By the end of the process, *rhlC* expression dropped down by half down to 2-fold.

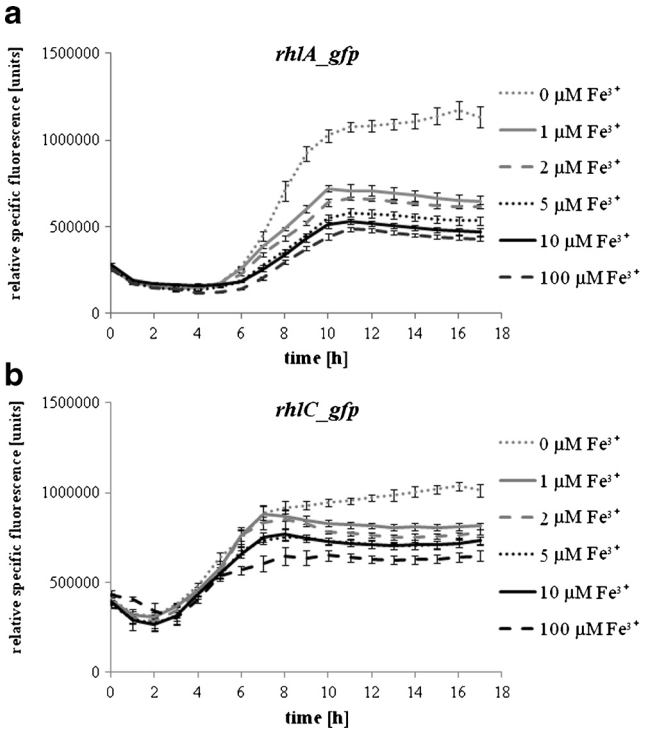

Fig. 5 Expression of *rhlA_gfp* and *rhlC_gfp* in planktonic cultures of *P. aeruginosa* PAO1 cultured in BM2 minimal medium containing different concentrations of Fe^{3+}. Expression of *rhlA_gfp* is shown on the *upper panel* (**a**), whereas *rhlC_gfp* is depicted on the bottom (**b**). *Dotted light grey line* represents cultures grown with 0 µM, *light grey line* 1 µM, *dashed light grey line* 2 µM, *dotted black line* 5 µM, *black line* 10 µM and *dashed black line* 100 µM Fe^{3+}. Measured gfp emission was correlated to the measured optical density to produce specific gfp emission

Besides the expression of rhamnolipid synthesis genes, expression of central QS components was investigated. Similar to the expression profiles of both *rhlA* and *rhlC*,

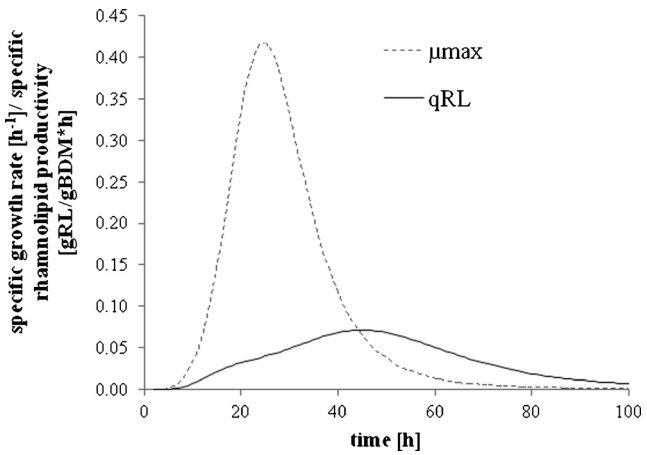

Fig. 6 Specific growth rate and specific rhamnolipid productivity by *P. aeruginosa* PAO1 during batch cultivation under iron-free conditions. The specific growth rate (µmax) is shown as *dashed line* and specific rhamnolipid productivity of total rhamnolipids (qRL) is shown as *solid black line*. For reasons of structure and clarity, a graphical presentation of the standard errors was omitted

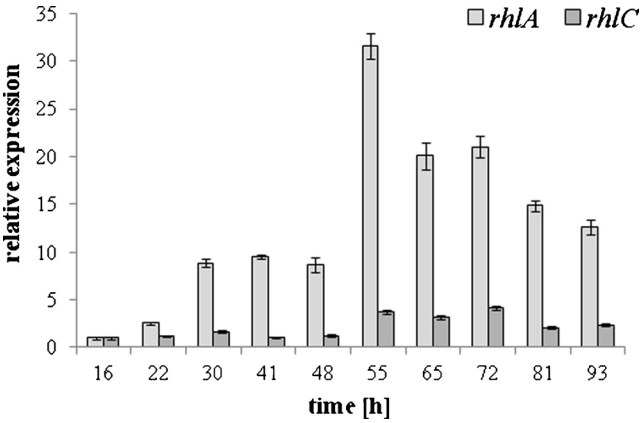

Fig. 7 Gene expression of rhamnosyltransferases 1 and 2 (*rhlA* and *rhlC*, respectively) in *P. aeruginosa* PAO1 during batch cultivation under iron-free conditions. The gene expression of *rhlA* and *rhlC* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at the specific times of cultivation

expression of the gene for the autoinducer synthetase, *lasI*, showed a strong up-regulation at 55 h of almost 6-fold (Fig. 8a). *LasI* expression then dropped by almost half after 65 h to reach another peak at 72 h of 4-fold. After that, *lasI* expression went down to 2-fold by the end of the cultivation. The gene for the corresponding regulator, *lasR*, did not show much regulation activity during the cultivation process, and its expression did not alter much over the entire time course compared to the reference point (Fig. 8a). Neither did the *pqs* system nor the expression of both *mvfR* (*pqsR*) and *pqsE* was even down-regulated compared to the reference (Fig. 8b). The *rhl* system on the other hand was activated already early during the process and *rhlR* expression was strongly up-regulated by 20-fold at 22 h of cultivation and remained more or less relatively high (Fig. 8c). Concurrent with *rhlA*, *rhlC* and *lasI*, *rhlR* expression reached a maximum expression of more than 35-fold at 55 h of cultivation. Even at the end of the process, *rhlR* was still up-regulated by 30-fold compared to the reference time point at 16 h. The gene for its corresponding C4-HSL synthetase, *rhlI*, showed a somewhat different expression profile and its expression increased steadily until it reached a peak at 72 h of cultivation, where it was expressed almost 3-fold compared to the reference (Fig. 8d). After that, *rhlI* expression dropped by almost half.

Furthermore, expression of various sigma factors was investigated. The gene for sigma factor for iron starvation, *pvdS*, was up-regulated by almost 5.5-fold at 30 h of cultivation (Fig. 9). After that, its expression steadily decreased to only 2-fold after 65 h of cultivation and went doubled again after 72 h. On the other hand, both the gene for the nitrogen limitation sigma factor, *rpoN*, and the stationary phase sigma factor, *rpoS*, did not show any up-regulation (data not shown). *RpoN* expression was even lower compared to the reference points at all time points over the entire time course of the process.

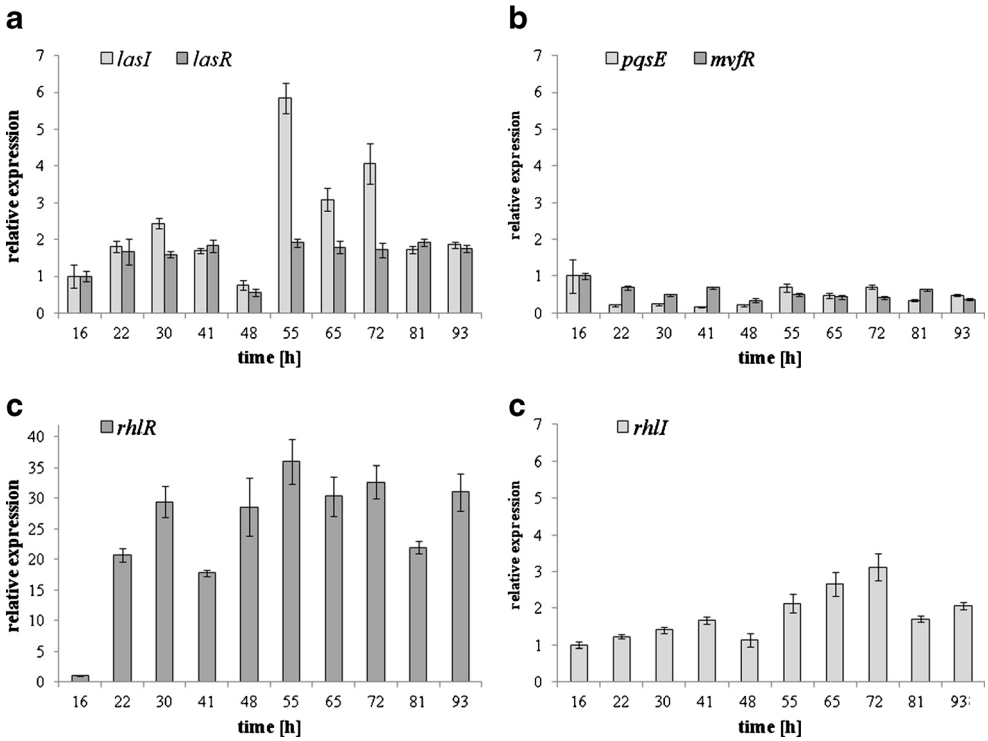

Fig. 8 Gene expression of the *las*, *pqs*, and *rhl* QS system in *P. aeruginosa* PAO1 during batch cultivation under iron-free conditions. The *las* QS system is shown on the *upper left* (**a**), the *pqs* QS system on the *upper right* (**b**), whereas the *rhl* system is shown on the *bottom* with the regulator on the *left* (**c**) and the synthase on the *right* (**d**). The gene expression of *lasI*, *lasR*, *pqsE*, *mvfR*, *rhlR* and *rhlI* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at the specific times of cultivation

Discussion

Rhamnolipids have become increasingly the focus of attention, yet the complex growth-dependent regulatory network behind rhamnolipid synthesis in *Pseudomonas aeruginosa* constitutes a major obstacle in the way to upscale production or the development of process control strategies. To develop new such strategies, the influence of ferric iron on growth and the expression of rhamnolipid synthesis genes, as well as other regulatory factors, was examined in a laboratory scale using *P. aeruginosa* PAO1 (DSM22644) in shake flask cultures.

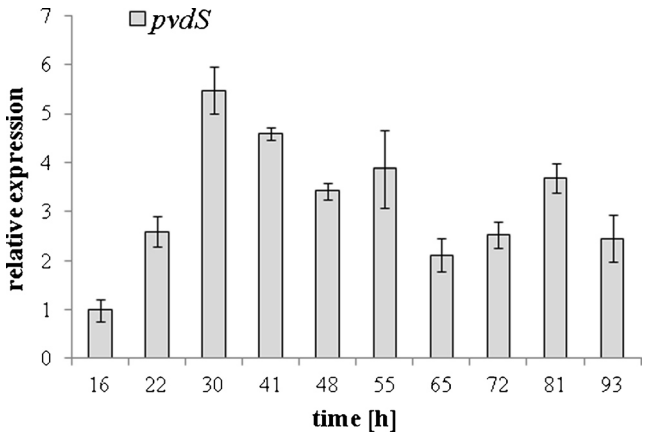

Fig. 9 Gene expression of the sigma factor PvdS in *P. aeruginosa* PAO1 during batch cultivation under iron deplete conditions. The gene expression of *pvdS* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as *bars* at the specific times of cultivation

Here, with concentrations between 0 and 2 μM Fe^{3+}, gene expression of both rhamnolipid synthesis genes, especially *rhlA*, was strongly up-regulated, which could be further confirmed by concurrent gfp assays and measurement of rhamnolipids in the culture supernatant. To investigate the potential of iron starvation to induce rhamnolipid production in upscaled bioprocesses, a small 2.2-L batch cultivation was conducted using the same *P. aeruginosa* strain grown in iron-free production medium. Analogous to the shake flask experiments, gene expression profiles were assessed using quantitative real-time PCR over the entire time course of the process.

Under iron-free cultivation conditions, *rhlA* expression was highest at 55 h into the process, whereas *rhlC* expression reached its maximum at 72 h. The maximum rhamnolipid production rate, however, was reached already after approximately 44 h. Compared to standard batch cultivation, the specific rhamnolipid production rate was generally lower (Schmidberger et al. 2013). This rather low production rate can be accounted for by the early *rhlA* activation at 30 h of cultivation, when *rhlA* expression was indeed high, but far below its maximum, which was reached much later in the process, when the culture settings possibly were unfavourable for rhamnolipid synthesis. By the time, when the genes for rhamnolipid synthesis reached their highest expression, the metabolism might already have been exhausted and essential precursors used otherwise.

Regarding the network that is involved in the regulation of rhamnolipid synthesis, divergent results were observable for the examined cultivations. In shake flasks, an upregulation of

the gene for the QS regulator of the *pqs* system *mvfR* (*pqsR*) was found under iron limitation. In the cultivation process, however, expression of *mvfR*, as well as the gene for its corresponding regulator protein, *pqsE*, remained low throughout the entire cultivation compared to the reference time point. The *rhl* system, on the other hand, especially expression of *rhlR*, was strongly increased during the entire batch process, whereas it was not in laboratory scale. This suggests that there might be different mechanisms of action for the activation of rhamnolipid synthesis in shake flasks and batch cultivation processes. *MvfR* codes for the transcriptional regulator MvfR (multiple virulence factor regulator; also known as PqsR), which is involved in the control of iron-regulated genes and hence the production of virulence factors, as well as the QS molecule PQS (Diggle et al. 2003; Deziel et al. 2004; Balasubramanian et al. 2013). Under certain culture conditions, i.e. iron-limiting conditions, expression of *mvfR* can be activated independently of the otherwise hierarchically higher ranked *las* system by PvdS through an iron starvation box in the *mvfR* promoter (Ochsner et al. 2002; Visca et al. 2002; Schuster and Greenberg 2006). As MvfR has been shown to regulate *rhl*-dependent genes without affecting the production of C4-HSL, it was suggested that *mvfR*/PQS and *rhl* QS function parallel to each other (Schuster and Greenberg 2006). Activation of rhamnolipid synthesis genes under iron starvation during shake flask cultivation might hence be mediated by PvdS-dependent *mvfR* activation rather than through *rhl* QS, as both *rhlR* and *rhlI* were not up-regulated as strongly as *mvfR*. However, both pathways might also act parallel to each other, or even amplify the common target that is rhamnolipid. In shake flask experiments using minimal medium, both the genes for the stationary phase sigma factor, *rpoS*, as well as for the nitrogen limitation sigma factor, *rpoN*, were up-regulated under iron-limiting conditions. Possibly, the strong activation of the rhamnolipid synthesis genes under iron limitation might be due to concerted actions of all three sigma factors, as expression of *pvdS* was also elevated. In the batch cultivation, both *rpoS* and *rpoN* were not up-regulated during the entire process, whereas *pvdS* expression started to increase early and continued to do so until mid-exponential phase of growth. As expression of *rhlA* and *rhlC* was highest much later than that, *pvdS* alone might not have been enough to activate the genes for rhamnolipid synthesis during batch cultivation under iron-limiting conditions at this early time point of the bioprocess.

Gfp measurements for monitoring the timing of *rhlA* and *rhlC* expression showed that under iron-limiting conditions, their expression started to increase much earlier than under normal or iron-replete conditions. During batch cultivation, however, rhamnolipid production did not start to increase until late exponential phase of growth, and the maximum specific rhamnolipid production rate was reached during early stationary growth phase, suggesting that there might be further regulatory or inhibitory mechanisms that counteract or prevent premature *rhlA*/*rhlC* activation. The synthesis of rhamnolipids is furthermore positively enhanced by a feedback loop. As product production was already reduced in the batch cultivation, this enhancing mechanism might have been out of action as well, thus leading to low production rates.

As the culture with which the fermenter was inoculated is already equipped with a certain stock of intracellular iron, it has yet to be evaluated when and if iron-limiting conditions de facto occurred during the bioprocess. How long a culture can live from these resources without further administration has yet to be demonstrated. Additionally, common reactors are for the most part made of steel. To minimise iron contamination, metallic surfaces of the stirring shaft, baffles and aeration tube were covered by shrink tubing. Elemental analyses using spectroscopic methods, such as optical emission spectroscopy, could be helpful to receive information about the actual iron situation during the cultivation process, both about the extracellular iron in the culture supernatant and the metabolised, intracellular iron. Consequently, an analogous, but void cultivation with sterilised, cell-free medium and subsequent evaluation of iron could be conducted. A very strong up-regulation under absolute iron-free conditions in shake flasks could be observed for the gene for the sensor protein, *bqsS*. The BqsS/BqsR two-component system has been described to sense extracellular ferrous iron, rather than ferric iron (Kreamer et al. 2012). However, iron depletion is independent of the administered oxidative form, as no iron means lack of both ferric and ferrous iron, respectively. During the batch process, the expression of *bqsS* was even down-regulated during the entire process. This could be a hint for that there might have been no actual iron-limiting conditions during cultivation. However, as *pvdS*, which is only expressed under iron limitation, showed up-regulation, this hypothesis may no longer tenable.

As there was a strong discrepancy between maximum rhamnolipid production rate and the timing of maximum expression of the two synthesis genes, a fed-batch, where the carbon source is administered at the time point of highest *rhlA* and *rhlC* expression, should be carried out in order to ensure that precursor shortage is no limiting factor. A shift in the ratio between rhamnolipid species towards dirhamnolipids could be observed in shake flask experiments by measuring their amount in the culture supernatant. This may be of major relevance for biotechnological production, since dirhamnolipid synthesis is to be favoured due to better downstream processing properties compared to monorhamnolipids. By controlling iron concentrations through adjusting the Fe^{3+} feed rate, so that the production of dirhamnolipids is favoured at all times during the entire process, might lead to substantially decreased concentrations of undesired monorhamnolipids. Possibly, the use of specific chelators that sequester ferric iron may be a suitable control strategy for causing a directed shift in rhamnolipid

species or transferring the process from growth stage into production stage. However, the capability of chelators to bind iron and prevent its uptake by *P. aeruginosa* has yet to be demonstrated. Additionally, if chelators may induce rhamnolipid synthesis remains to be elucidated as well.

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v. Iron Sequestration and Rhamnolipid Production in *Pseudomonas aeruginosa* PAO1

Most studies have focused on targeting or chelating iron with regard to the reduction of biofilm formation, as chronic biofilm-like infections by *P. aeruginosa* are often the major cause of infections in cystic fibrosis (CF) patients (Hunter et al. 2013; Smith et al. 2013). Iron has been demonstrated to function as a signal for biofilm development in *P. aeruginosa* (Banin et al. 2005). Iron sequestration by the means of chelators such as the mammalian iron chelator lactoferrin, was able to prevent biofilm maturation of a thin cell layer of *P. aeruginosa* (Singh et al. 2002). Furthermore, it has been demonstrated in the mucoid *P. aeruginosa* PA17 that rhamnolipids are responsible for so-called seeding dispersal, one of the three major ways for cell detachment, which can mostly be seen in aging biofilms (Skaar 2010; Wang et al. 2013). Additionally, rhamnolipid synthesis was shown to be increased under iron-limiting conditions, while twitching motility and a planktonic lifestyle were also enhanced (Glick et al. 2010; Schmidberger et al. 2014). In *P. aeruginosa*, iron starvation has recently been described to be mediated by the two-component system BqsS-BqsR that senses extracellular ferrous iron (Kreamer et al. 2012). Mutations in BqsS-BqsR in *P. aeruginosa* PAO1 resulted in a massive reduction in rhamnolipid synthesis, as well as reduced C4-HSL and PQS signal production (Balasubramanian et al. 2013). Other studies have shown that there are also components, namely the virulence and quorum-sensing regulator, VqsR, that link iron to quorum sensing (Cornelis et al. 2004).

As there certainly is a close link between iron, biofilm formation and rhamnolipid synthesis, targeting iron availability could be a useful approach to an optimised and targeted process control strategy for the production of rhamnolipids. By controlled induction of iron limiting conditions by sequestration through specific chelators at any time during the cultivation, the bioprocess might be directly and purposely transferred from growth stage after sufficient biomass formation into production stage. Rhamnolipid synthesis might hence be switched on selectively. This hypothesis is depicted in figure 1. By administration of specific iron chelators that sequester free iron from the culture medium, limiting conditions could be created artificially. Consequently,

production of rhamnolipids might be induced due to the introduced iron limitation leading to a premature and enhanced rhamnolipid production.

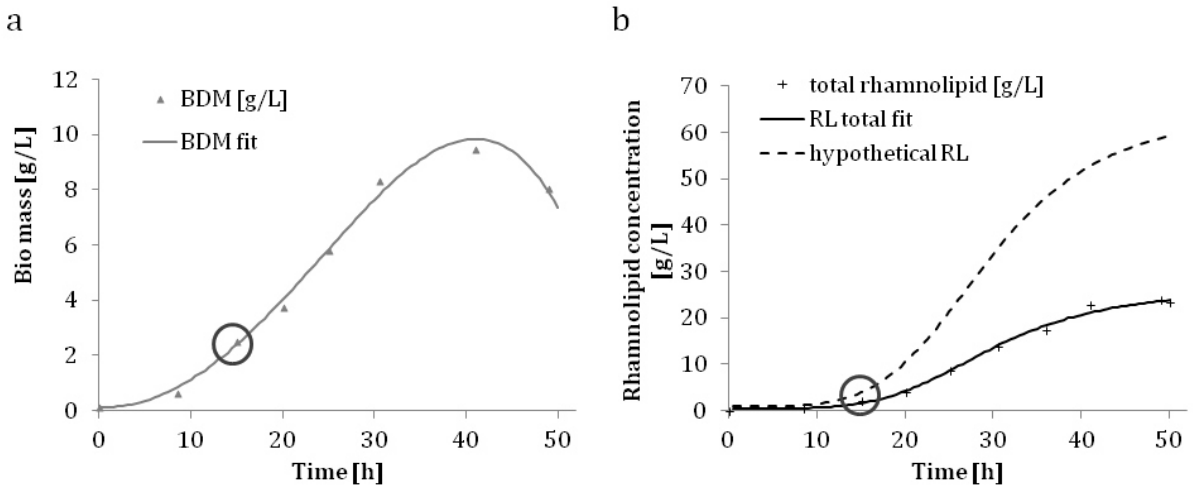

Fig. 1 Hypothesis on iron sequestration and rhamnolipid production. (a). Measured bio mass is indicated by triangles, while the solid line represents a logistic fit based upon the measured data. Rhamnolipid production is shown on the right panel (b). Measured total rhamnolipids are indicated by plus signs, while the solid line represents a logistic fit based on the measured data. The hypothetical increased rhamnolipid production is shown as dashed line. Arrows indicate the time point of chelator administration

In this chapter, the effect on the regulatory network behind rhamnolipid synthesis was examined in response to sequestering Fe^{3+} from the culture medium by specific iron chelators.

As chelators, the following natural and synthetic Fe^{3+} chelation compounds were tested for their ability to induce iron starvation and as a consequence rhamnolipid synthesis in *P. aeruginosa* PAO1 in shake flask cultures: acetohydroxamic acid, picolinic acid, 2,2'-bipyridine, lactoferrin, ethylenediaminetetra-acetic acid, and FerroZine™. An overview of the chemical structures of all chelators is given in figure 2. The amino acid sequence of bovine lactoferrin can be derived from Goodman and Schanbacher (Goodman et al. 1991).

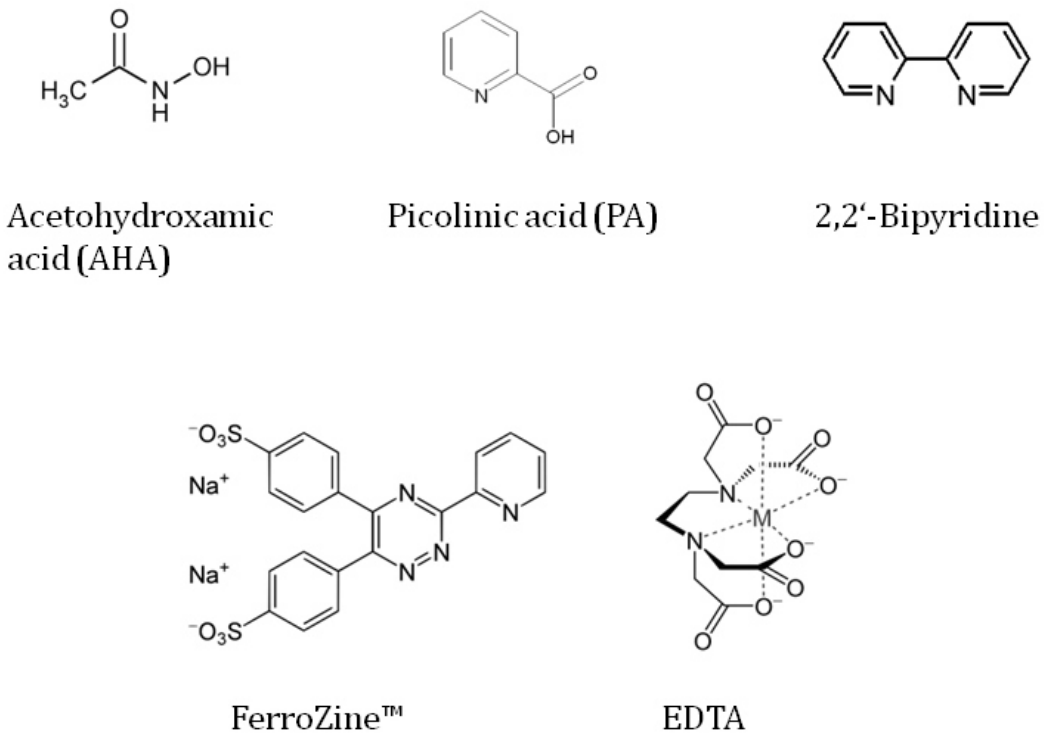

Fig. 2 Specific iron chelators. Chemical **s**tructures of iron chelators used in this study

Acetohydroxamic acid (AHA) is a compound that is structurally similar to urea. It is however not cleaved by bacterial urease and was demonstrated to irreversibly, completely and non-competitively inhibit the enzyme (Fishbein et al. 1965). Picolinic acid (PA) belongs to the group of natural chelators and is produced as a byproduct of the human tryptophan catabolism (Bosco et al. 2003). Both AHA and PA were found to exhibit anti-biofilm effects against lab as well as clinical strains of *P. aeruginosa* PA14 (Musk et al. 2008). The human immune protein lactoferrin has been shown to substantially decrease biofilm formation under oxic conditions (Singh 2004; Banin et al. 2005; Banin et al. 2006). 2,2'-bipyridine is a specific iron chelator that is often used to manipulate iron bioavailability in culture media (Bollinger et al. 2001; Frangipani et al. 2013). FerroZine™ (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid monosodium salt hydrate) is an organic substance, which can be utilised for the spectrometric detection of iron in waters or human serum (Viollier et al. 2000).

Ethylenediaminetetraacetic acid (EDTA) is commonly used in laboratories to sequester metal ions, such as Ca^{2+} or Fe^{3+}, and was furthermore shown to decrease biofilm growth and enhance the efficacy of conventional antibiotics (Banin et al. 2006; Moreau-Marquis et al. 2009).

Influence of specific iron chelators on the growth of *P. aeruginosa* PAO1

To determine that the used chelators are non-toxic or impair bacterial growth, growth curves were assessed prior to gene expression analyses. In doing so, cultures were either grown in standard BM2, without addition of iron or with administration of specific chelators in a stoichiometrical proportion of iron to chelator of 1:50.

Cultures that were held in standard BM2 showed normal logistic growth, while cultures that were grown without addition of ferric iron showed impaired growth (Fig. 3a and 3b). Those cultures reached a maximum optical density (OD) of almost 0.7 by the end of the cultivation after 20 hours and cultures grown under standard conditions reached an OD of more than 1.1. Administration of the chelators PA, AHA, lactoferrin, and FerroZine™ did not have an effect on bacterial growth and these cultures reached an equally high OD as cultures grown in standard BM2 medium. EDTA on the other hand caused delayed and impaired growth, so that these cultures started to grow time-displaced by almost 2 hours after an extended lag-phase and reached a maximum OD of approximately 0.9 by the end of the cultivation. Strong impairment of growth was observed in cultures grown in addition of 2,2'-bipyridine and there was hence no bacterial growth observable. On this account, 2,2'-bipyridine was discarded as potential chelator for gene expression analyses since there was no sufficient biomass production and consequently, not enough amount of total RNA could be isolated.

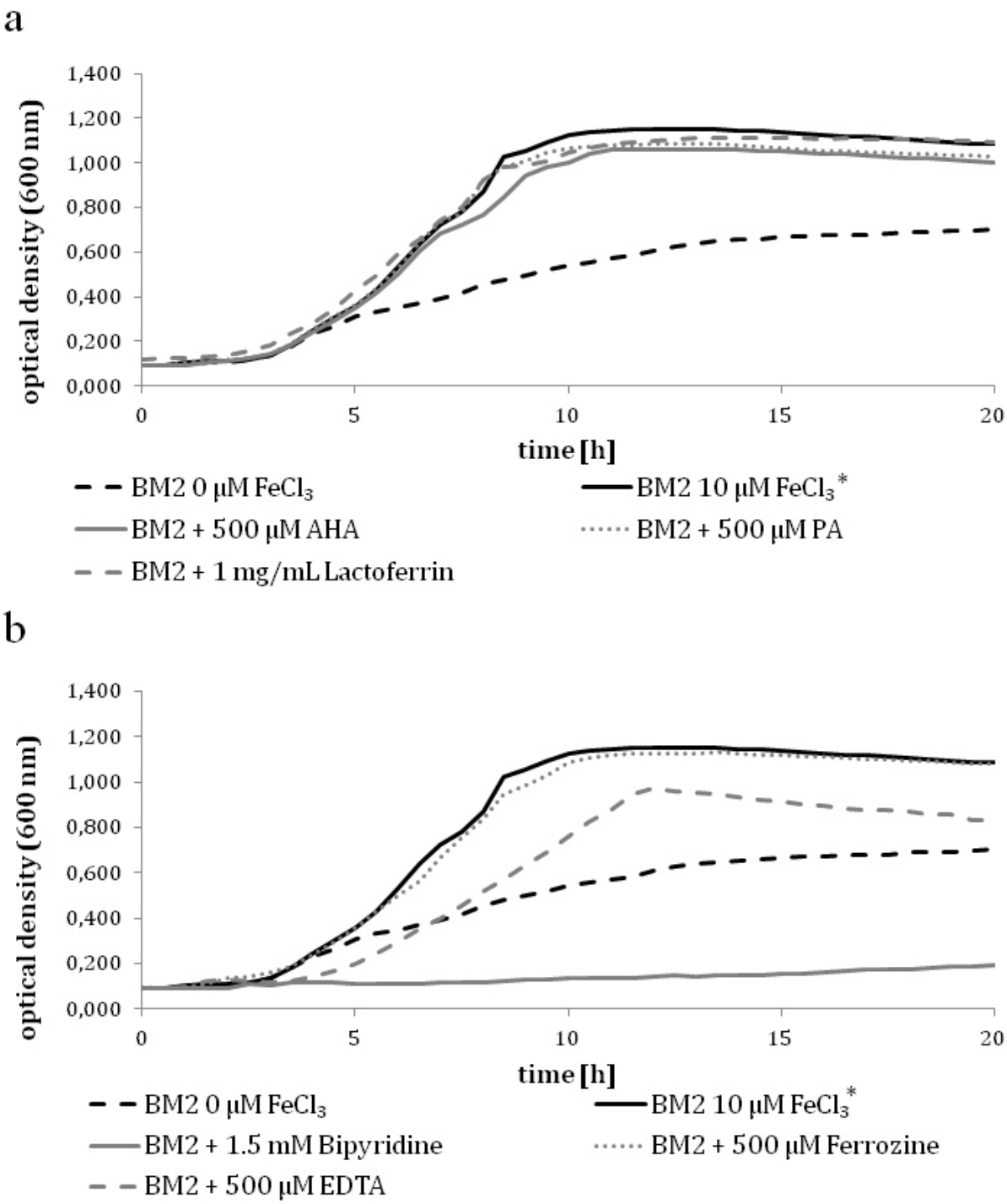

Fig. 3 Growth of *P. aeruginosa* PAO1 in response to iron chelating compounds. Dashed black line represents cultures grown in BM2 medium without addition of $FeCl_3$ and solid black line represents standard BM2 with 10 µM $FeCl_3$, which is indicated by the asterisk. (a) Grey lines indicate cultivations in standard BM2 medium with supplemented iron chelators, whereas the solid grey line represents administration of acetohydroxamic acid (AHA), dotted grey line represents picolinic acid (PA), and dashed grey line represents lactoferrin. (b) Solid grey line represents administration of bibyridine, dotted grey line represents FerroZine™, and dashed grey line represents ethylenediaminetetraacetic acid (EDTA)

Influence of specific iron chelators on rhamnolipid synthesis and its regulatory network in *P. aeruginosa* PAO1

Administration of the chelators immediately prior to cultivation did not have an effect on the expression of rhamnolipid synthesis genes (data not shown), which is why freshly prepared BM2 medium was incubated with a given chelator several days prior to cultivation to make sure that all iron was chelated as shown in figure 4. After inoculation with *P. aeruginosa* PAO1, samples taken for gene expression analyses after 6 hours of incubation. As a reference, standard BM2 was chosen and relative gene expression was determined in relation to the expression of genes in these cultures.

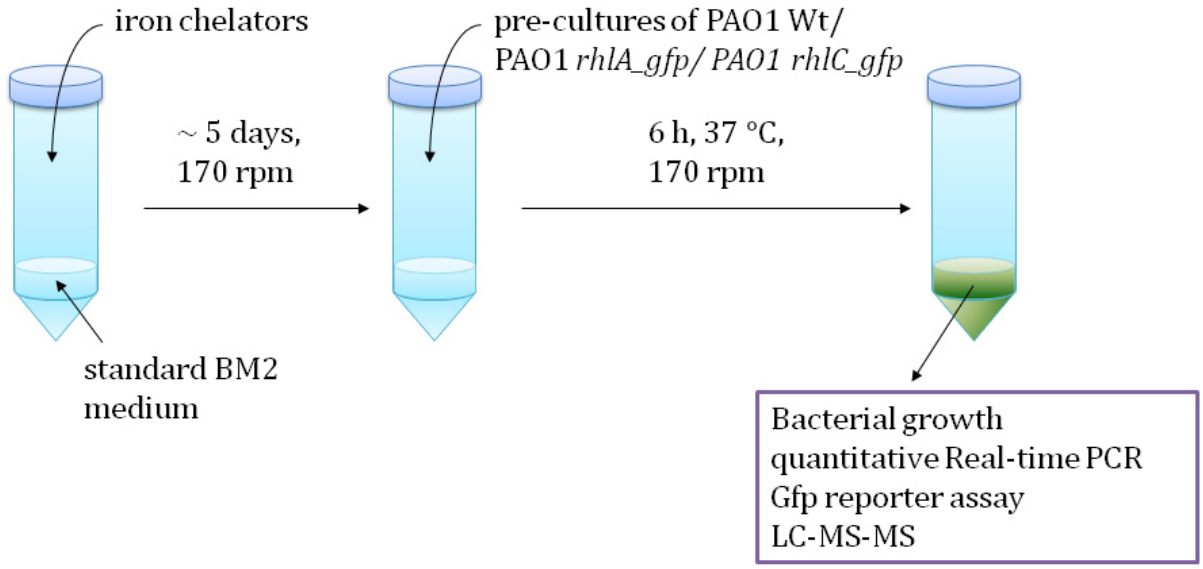

Fig. 4 Experimental set-up for the sequestration of iron by specific iron chelators. Freshly prepared BM2 medium was incubated with a given chelator in a stoichiometry of 1:50 several days prior to cultivation to make sure that all iron was chelated. The prepared media were then inoculated with wildtype *P. aeruginosa* PAO1 or *rhlA_gfp* and *rhlC_gfp* reporter strains. Samples for gene expression analyses as well as for rhamnolipid quantification by LC/MS/MS were taken in mid-exponential phase after 6 hours of incubation. Bacterial growth as well as gfp fluorescence emission was assessed in microtiter plates using the same pre-cultures

The OD of the respective cultures was determined concurrently during sampling. While the culture that was grown under standard conditions had reached an OD of 0.8, cultures that were grown without the addition of iron reached an OD of 0.6 (Fig 5). In chelator-treated cultures, ODs were either similar to that in standard cultures, as it was the case for PA and FerroZine™. In cultures that were treated with AHA and lactoferrin, the OD was 1.0. EDTA on the other hand caused an OD of 0.2, which was much lower than under standard conditions.

Under iron depleted conditions, expression of the genes for the two rhamnosyltransferases, *rhlA* and *rhlC*, were strongly up-regulated by more than 20-fold, and approximately 5-fold respectively, compared to standard conditions (Fig. 5). In response to administration of the chelators, no up-regulation could be observed for all chelators for both genes, except for acetohydroxamic acid. Administration of AHA did cause a slight increase in expression of *rhlA* and *rhlC* by 3- and 2-fold respectively. However, this positive effect could not be verified by a concurrent gfp-assay with *rhlA_gfp* and *rhlC_gfp* fusion vectors, where the same cultivation conditions were investigated. Under iron-free BM2 medium, both *rhlA_gfp* and *rhlC_gfp* expression was increased. Administration of the chelators AHA, lactoferrin, PA, and ferroZine elicited a similar expression trend for both *rhlA_gfp* and *rhlC_gfp* compared to cultures with standard BM2 medium without the addition of chelators (Fig. 6a, 6b, 7a, and 7b). Alone EDTA showed an effect on *rhlA_gfp* expression. Here, gfp fluorescence started to increase slightly time-displaced compared to cultures that were grown under standard conditions. In response to EDTA administration, *rhlA_gfp* expression also reached a higher plateau after transition to stationary phase than it did in standard BM2 medium.

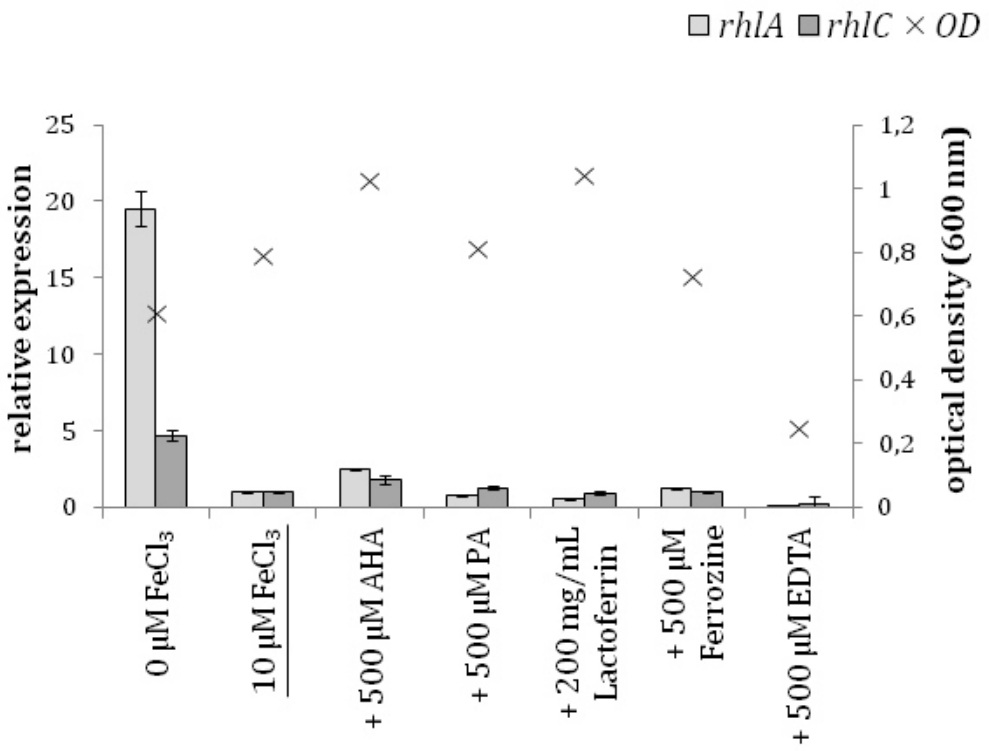

Fig. 5 Gene expression of rhamnosyltransferases 1 and 2 (*rhlA* and *rhlC,* respectively) in *P. aeruginosa* PAO1 in response to iron chelating compounds. The gene expression of *rhlA* and *rhlC* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as bars with supplementation of different iron chelators during shake flask experiments. Prior to incubation, medium was treated with a given chelator and samples were then collected at mid-exponential phase after 6 hours of cultivations. Expression of genes in standard BM2 Medium with 10 µM $FeCl_3$ was used as a reference for the calculation of expression ratios and is therefore underlined

RhlC_gfp expression on the other hand also showed delayed expression, its maximum expression however was as high as under standard conditions. 2,2'-Bypridine did not show relative specific gfp fluorescence during the entire period of the cultivation for neither *rhlA* nor *rhlC,* which is mainly due to the major growth impairment caused by 2,2'-bipyridin on the growth of *P. aeruginosa* PAO1.

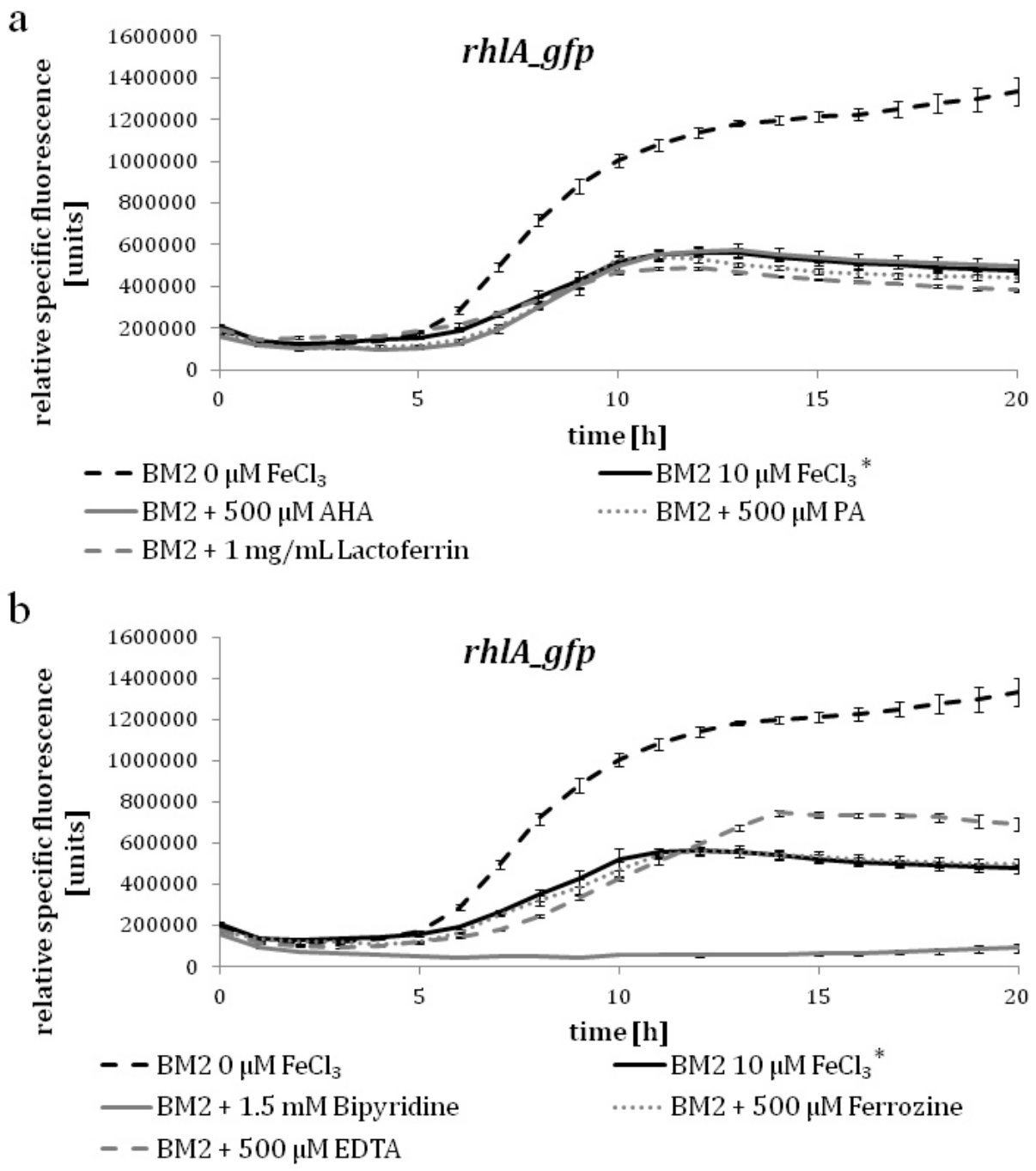

Fig. 6 Expression of *rhlA_gfp* in planctonic cultures of *P. aeruginosa* PAO1 in standard BM2 medium containing specific iron chelating compounds. Dashed black line represents cultures grown in BM2 medium without addition of $FeCl_3$ and solid black line represents standard BM2 with 10 µM $FeCl_3$, which is indicated by the asterisk. (a) Solid grey line represents administration of acetohydroxamic acid (AHA), dotted grey line represents picolinic acid (PA), and dashed grey line represents lactoferrin. (b) Solid grey line represents administration of bibyridine, dotted grey line represents FerroZine™, and dashed grey line represents ethylenediaminetetraacetic acid (EDTA). Measured gfp emission was correlated to the measured optical density at 600 nm to produce specific gfp emission

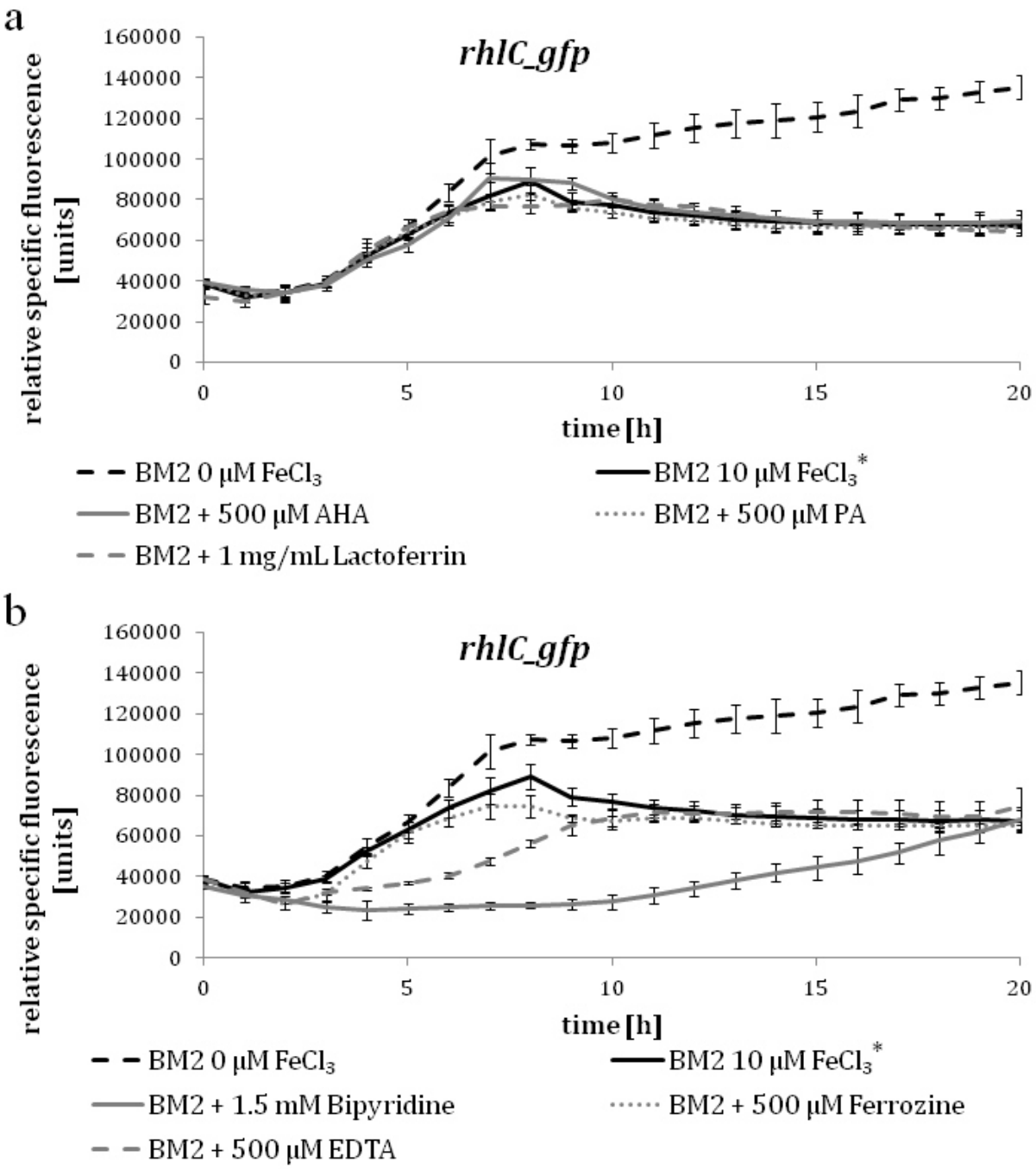

Fig. 7 Expression of *rhlC_gfp* in planctonic cultures of *P. aeruginosa* PAO1 in standard BM2 medium containing specific iron chelating compounds. Dashed black line represents cultures grown in BM2 medium without addition of $FeCl_3$ and solid black line represents standard BM2 with 10 µM $FeCl_3$, which is indicated by the asterisk. (a) Solid grey line represents administration of acetohydroxamic acid (AHA), dotted grey line represents picolinic acid (PA), and dashed grey line represents lactoferrin. (b) Solid grey line represents administration of bibyridine, dotted grey line represents FerroZine™, and dashed grey line represents ethylenediaminetetraacetic acid (EDTA). Measured gfp emission was correlated to the measured optical density at 600 nm to produce specific gfp emission

Production of rhamnolipids was also determined in cell-free culture supernatant samples, which were taken during mid-exponential phase after 6 hours of cultivation using liquid chromatography tandem mass spectrometry (LC/MS/MS). However, rhamnolipids could only be detected in cultures that were cultivated without an addition of iron (Supplemental Data, Fig. S13). In the cultures with standard BM2, as well as in those that were given specific chelators, no rhamnolipid production could be measured at this stage of cultivation.

Another focus of attention has been the greater regulatory network of rhamnolipid synthesis. Therefore, central components of the three QS systems, as well as different sigma factors have been under investigation using qPCR. However, neither of the investigated chelators showed a significantly positive effect on the regulatory network. The figures with the gene expression data can be found as supplemental figures.

Briefly, all three QS systems showed a positive response to iron deplete cultivation conditions. Both the *rhl* and *pqs* system was up-regulated by approximately 2- to 4-fold, whereas the *las* QS system was up-regulated by 2- to 3-fold (Supplemental Data, Fig. S14, Fig. S15, and Fig. S16). The specific chelators were not able to cause an up-regulation of the three QS systems with one exception. AHA caused a slight up-regulation of the rhl QS system by 2-fold (Fig. S14). Additionally, the *las* system was marginally up-regulated by almost 2-fold in response to most chelators (Fig. S15). Alone EDTA caused a strong down-regulation of all three QS systems.

Concluding, several sigma factors have been under investigation. First, the gene for the sigma factor for iron limitation, *pvdS*, was examined. Under iron-free conditions, *pvdS* was strongly up-regulated by more than 30-fold compared to standard conditions (Supplemental Data, Fig. S16a). It was also up-regulated by a minor extent in cultures that were treated with AHA and PA that is by 7-fold and 4-fold respectively, whereas the remaining chelators had a negative effect. *RpoN*, the gene for the sigma factor for nitrogen limitation, was neither up- nor down-regulated in the all the examined cultures, including that without iron in the medium (Supplemental Data, Fig. S16b). The gene for the stationary phase sigma factor, *rpoS*, was only up-regulated in cultures that were grown without the addition of iron, i.e. by more than 3-fold (Supplemental Data,

Fig. S16a). In all cultures that were treated with chelators, expression of *rpoS* remained approximately as low as under standard conditions.

To sum up, none of the chelators under investigation were able to elicit an increased expression of the rhamnolipid synthesis genes, nor could they induce an enhanced production. Additionally, the regulatory network around rhamnolipid synthesis remained unresponsive to administration of the specific iron chelators.

P. aeruginosa is known to produce compounds itself that specifically target iron. Those so-called siderophores mediate the uptake of iron into the bacterial cell. However, complex stability of siderophores and iron has to be taken into consideration, as iron might be scavenged from chelators that have weaker binding affinities to iron. Furthermore, *P. aeruginosa* might be able to take up chelators and chelator-bound iron. Uptake might even be enhanced as it is for siderophore-bound iron. Gene expression data showed that under iron depletion, the gene for the sigma factor for iron limitation, *pvdS*, was strongly up-regulated, whereas it was not in response to the chelators. This confirms the hypothesis that the chelators were indeed not able to induce an iron scarce environment in the cultures. Furthermore the chelating compounds have a yet unknown effect on the physiology of *P. aeruginosa* and might interfere with other metabolomic pathways.

As it was previously shown, iron depletion clearly had a positive influence on rhamnolipid synthesis in *P. aeruginosa* (Schmidberger et al. 2014). Sequestration of free iron from the production medium by specific chelators might still be an interesting option for optimisation. However, from a downstream processing point of view, administration of additional compounds would mean additional purification steps, which ultimately leads to decreased product yields and increased product costs. Furthermore, addition of supplementary compounds increase process costs, which is also not to be favoured.

VII. Conclusion and Outlook

In this thesis, the regulatory network behind rhamnolipid production in *Pseudomonas aeruginosa* PAO1 was to be characterised on a molecular basis in various multi-scaled cultivation scenarios.

The results can be summarised as follows:

- Quantitative PCR (qPCR) was successfully employed to create a 'molecular toolbox' with 23 genes of the regulatory network of rhamnolipid synthesis
- Different optimisation strategies have been examined and developed to find new ways to improve rhamnolipid production. Iron depletion proved to be a potential and promising trigger for rhamnolipid production in small-scale shake flask experiments and its influence has been characterised on a molecular and metabolomic level
- A large-scale standard batch process for rhamnolipid production was analysed using the molecular toolbox over the entire time course of the cultivation. Crucial points of regulation could be identified
- First scale-up attempt in a larger-scale bioreactor using iron-free production medium failed to reproduce a positive effect on rhamnolipid production observed in shake flasks
- Hypothetical induction of rhamnolipid production by introducing artificial iron-limiting conditions through sequestration of free iron by specific chelating compounds remains to be demonstrated

Iron depletion had a pronounced positive effect on rhamnolipid production in small-scale shake flasks. However, in the larger-scale bioreactor, this was not the case. This suggests that there might be problems with the transfer from the small- to the larger-scale cultivation, which need to be addressed to fully exploit the high potential that iron limitation has on the improvement of rhamnolipid production. One hypothesis could be that there were no actual iron-free conditions present in the bioreactor as it is partly made of metal. Shrink tubing was applied to cover those parts. However, this may

not have been enough to prevent the solubilisation of iron ions into the production medium.

In future, the obtained gene expression data for the standard and the alternated batch cultivation will be integrated into the process model, which has been established together with the Fraunhofer Institute of Optronics, System Technologies and Image Exploitation (IOSB) and the Department of Technical Biology of the Karlsruhe Institute of Technology to be able to describe the process more accurately and make even more precise predictions.

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IX. Appendices

i. Abbreviations

Abbreviation	
μ	Growth rate
μ_{max}	Maximum growth rate
3-oxo-C12-HSL	N-(3-oxododecanoyl)-L-homoserine lactone
AHA	Acetohydroxamic acid
AHL	N-acylated homoserine lactone
AQ	2-alkyl-4-quinolone
BDM	Bio dry mass
BM2	Basal Medium 2
C4-HSL	N-butyryl-L-homoserine lactone
cDNA	Complementary DNA
CF	Cystic Fibrosis
C_t	Cycle threshold
DNA	Deoxyribonucleic Acid
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
FDA	Federal Drug Agency
Gfp	Green fluorescence protein
HAA	3-(3-hydroxyalkanoyloxy)alkanoic acid
HHQ	2-heptyl-4-quinolone
HPLC	High performance liquid chromatography
IOSB	Institute of Optronics, System Technologies and Image Exploitation
LB	Lysogeny broth; Luria Bertani
LC/MS/MS	Liquid Chromatography Tandem Mass Spectrometry

Mbp	Million base pairs
n.d.	Not detected
ORF	Open reading frame
PA	Picolinic acid
PCA	Protocatechuic acid
PCR	Polymerase Chain Reaction
PQS	*Pseudomonas* Quinolone Signal
$q_{RL, spec}$	Specific RL production rate
QS	Quorum Sensing
qscR	Quorum Sensing Control Repressor
RL	Rhamnolipid
RNA	Ribonucleic Acid
rpm	Rounds per minute
RT-PCR	Real-Time PCR
undet.	Undetermined

ii. Supplemental Data

Table ST1 Mean Ct values for PCR efficiency calculation

log cDNA	*algR*	*bqsS*	*bqsR*	*fab*	*fur*	*gacA*	*lasI*
-5	undet.	undet.	undet.	undet.	undet.	undet.	undet.
-4	35.73	undet.	undet.	33.25	undet.	undet.	34.47
-3	31.56	undet.	32.57	29.31	35.67	33.04	31.32
-2	28.59	36.00	32.03	25.96	34.09	29.07	26.67
-1	25.29	33.74	29.07	23.13	30.75	25.96	23.43
0	22.14	29.78	25.70	19.09	26.12	22.06	20.15

Table ST2 Mean Ct values for PCR efficiency calculation

log cDNA	*lasR*	*lipC*	*mvfR*	*pqsE*	*pvdS*	*qscR*	*rhlA*
-5	37.00	undet.	undet.	undet.	34.87	37.38	undet.
-4	34.78	38.68	37.00	undet.	29.66	undet.	undet.
-3	30.81	35.53	33.22	undet.	undet.	31.44	35.14
-2	28.45	31.89	30.49	33.94	26.59	28.20	31.89
-1	26.29	29.10	27.68	32.91	23.56	24.71	28.72
0	20.87	26.00	24.21	29.80	20.16	21.00	24.81

Table ST3 Mean Ct values for PCR efficiency calculation

log cDNA	*rhlC*	*rhlI*	*rhlR*	*rpoD*	*rpoN*	*rpoS*	*rsaL*
-5	38.00	undet.	undet.	undet.	undet.	undet.	undet.
-4	39.00	undet.	34.13	34.47	34.67	36.68	32.55
-3	undet.	34.23	31.71	30.93	31.59	33.37	31.55
-2	32.60	30.81	28.27	27.94	28.82	30.74	28.81
-1	27.51	28.11	25.12	25.51	25.75	28.54	25.76
0	23.78	24.52	21.45	21.33	21.73	23.08	22.32

Table ST4 Mean Ct values for PCR efficiency calculation

log cDNA	*rsmA*	*vfR*
-5	36.74	undet.
-4	33.84	30.25
-3	30.50	27.38
-2	26.30	23.98
-1	23.05	20.49
0	19.57	17.24

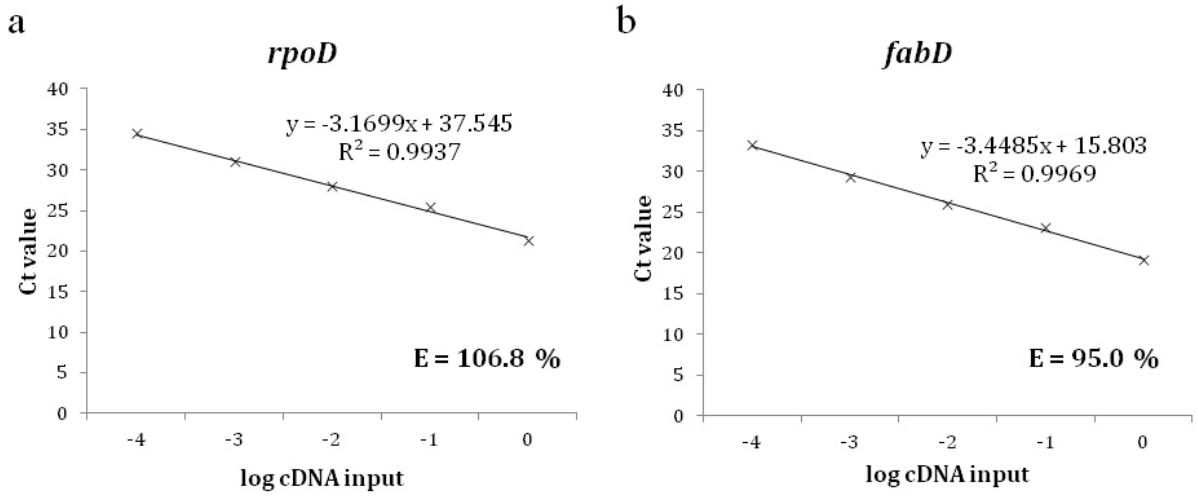

Fig. S1 PCR efficiency of the housekeeping genes *rpoD* and *fabD* in *P. aeruginosa* PAO1. PCR efficiencies of *rpoD* (a) and *fabD* (b) were determined using the serial dilution method and standard curves were generated by plotting the cycle at the fluorescence threshold (C_t value) against the log of target DNA concentration. The efficiency in percent was calculated from the slope of the regression curve and is indicated by *E* in the graph

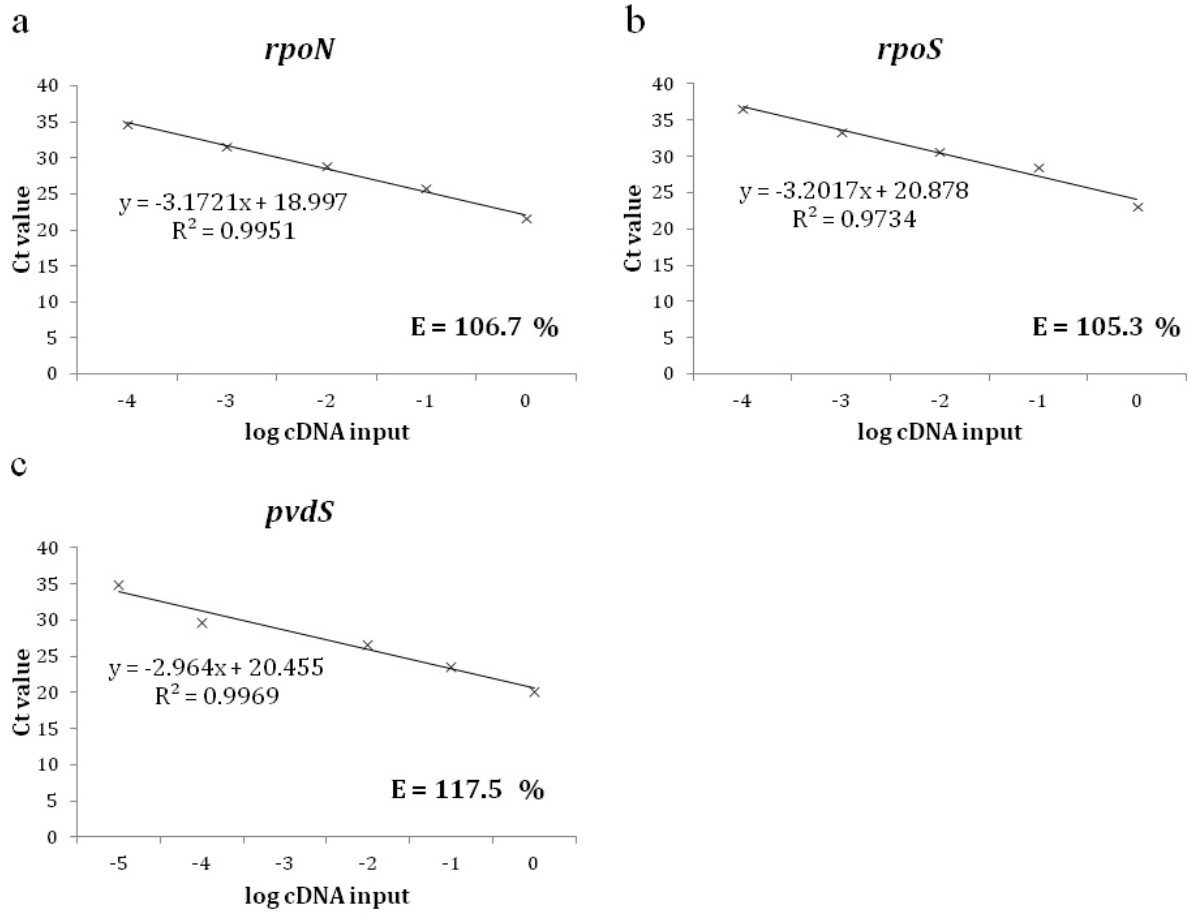

Fig. S2 PCR efficiency of the genes for the sigma factors RpoN, RpoS, and PvdS in *P. aeruginosa* PAO1. PCR efficiencies of *rpoN* (a), *rpoS* (b), and *pvdS* (c) were determined using the serial dilution method and standard curves were generated by plotting the cycle at the fluorescence threshold (C_t value) against the log of target DNA concentration. The efficiency in percent was calculated from the slope of the regression curve and is indicated by *E* in the graph

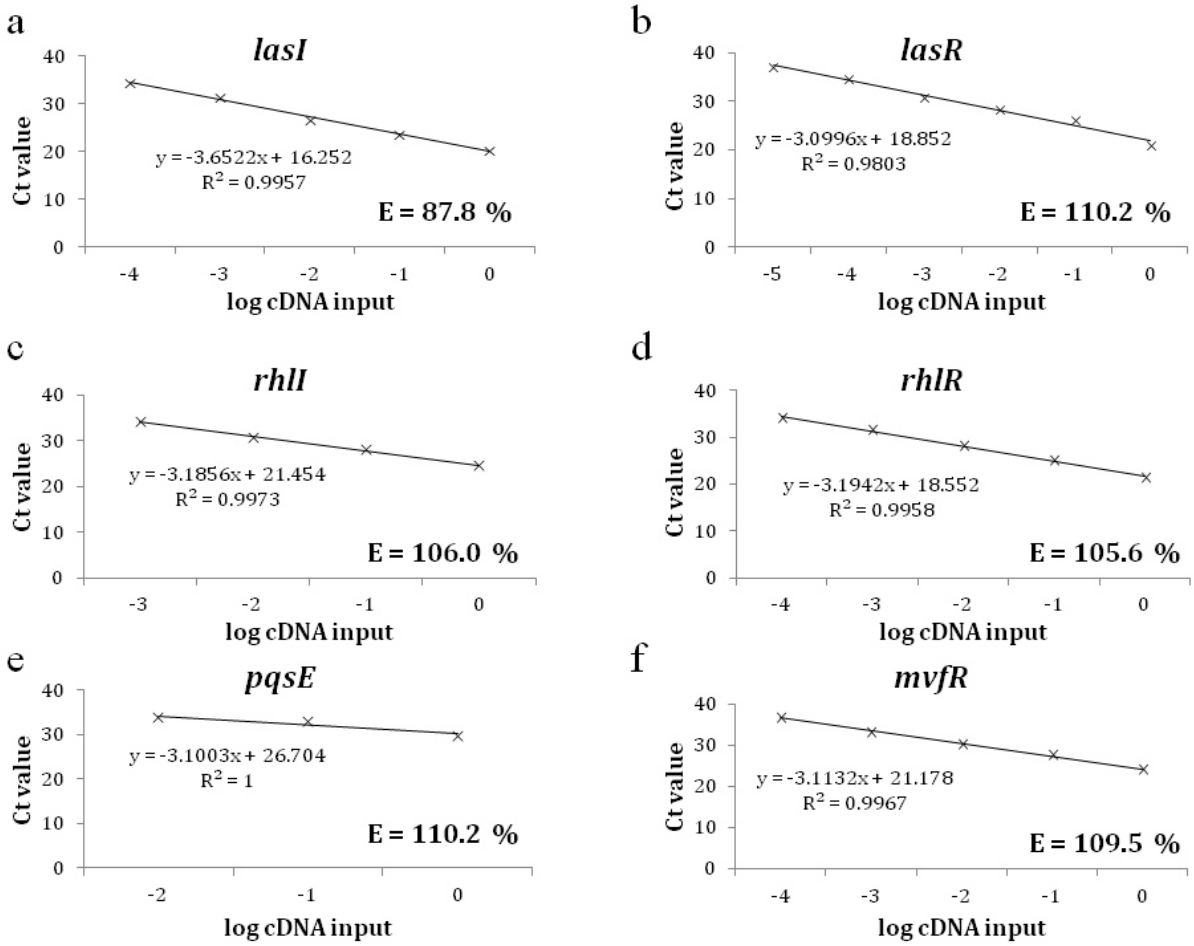

Fig. S3 PCR efficiency of the genes for the *las*, *rhl*, and *pqs* QS systems in *P. aeruginosa* PAO1. PCR efficiencies of *lasI* (a), *lasR* (b), *rhlI* (c), *rhlR* (d), *pqsE* (e), and *mvfR* (f) were determined using the serial dilution method and standard curves were generated by plotting the cycle at the fluorescence threshold (C_t value) against the log of target DNA concentration. The efficiency in percent was calculated from the slope of the regression curve and is indicated by *E* in the graph

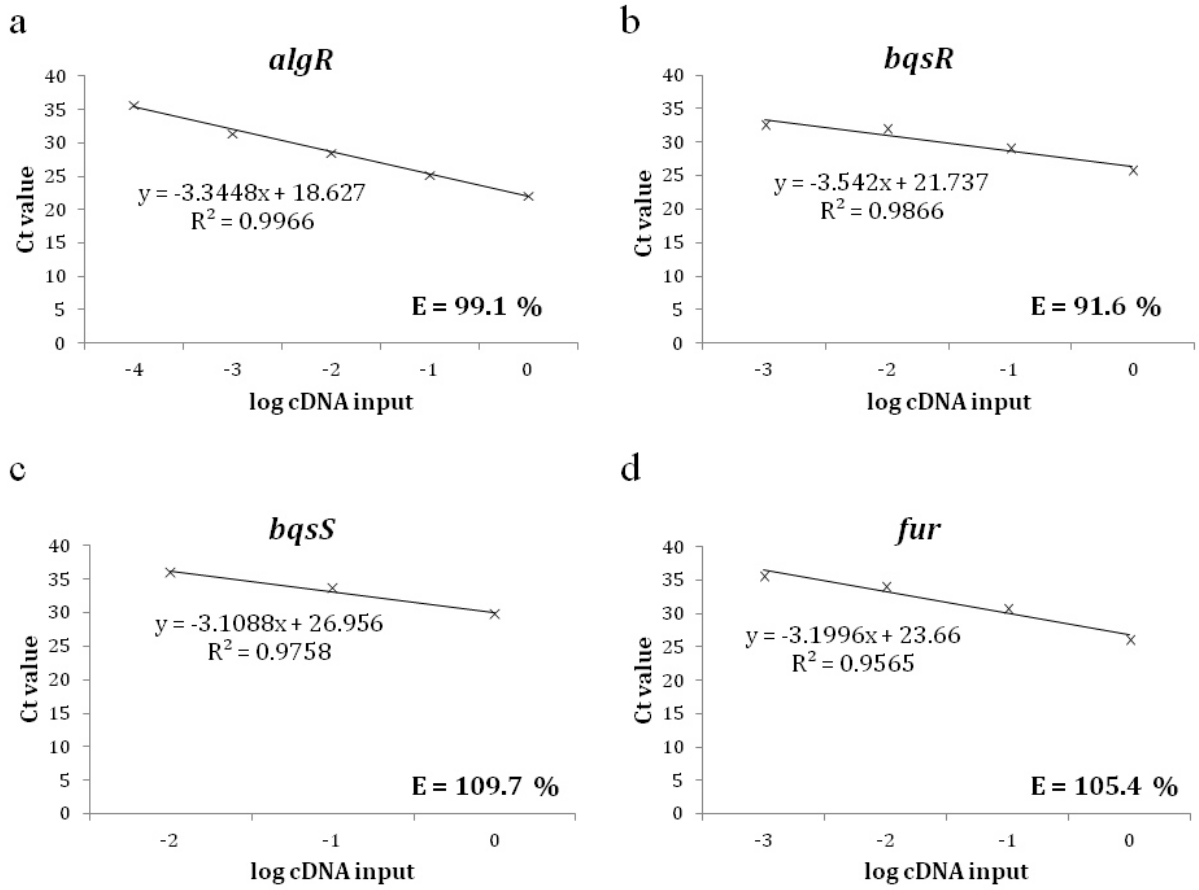

Fig. S4 PCR efficiency of the genes for regulatory factors AlgR, BqsR, BqsS, and Fur in *P. aeruginosa* PAO1. PCR efficiencies of *algR* (a), *bqsR* (b), *bqsS* (c), and *fur* (d) were determined using the serial dilution method and standard curves were generated by plotting the cycle at the fluorescence threshold (C_t value) against the log of target DNA concentration. The efficiency in percent was calculated from the slope of the regression curve and is indicated by *E* in the graph

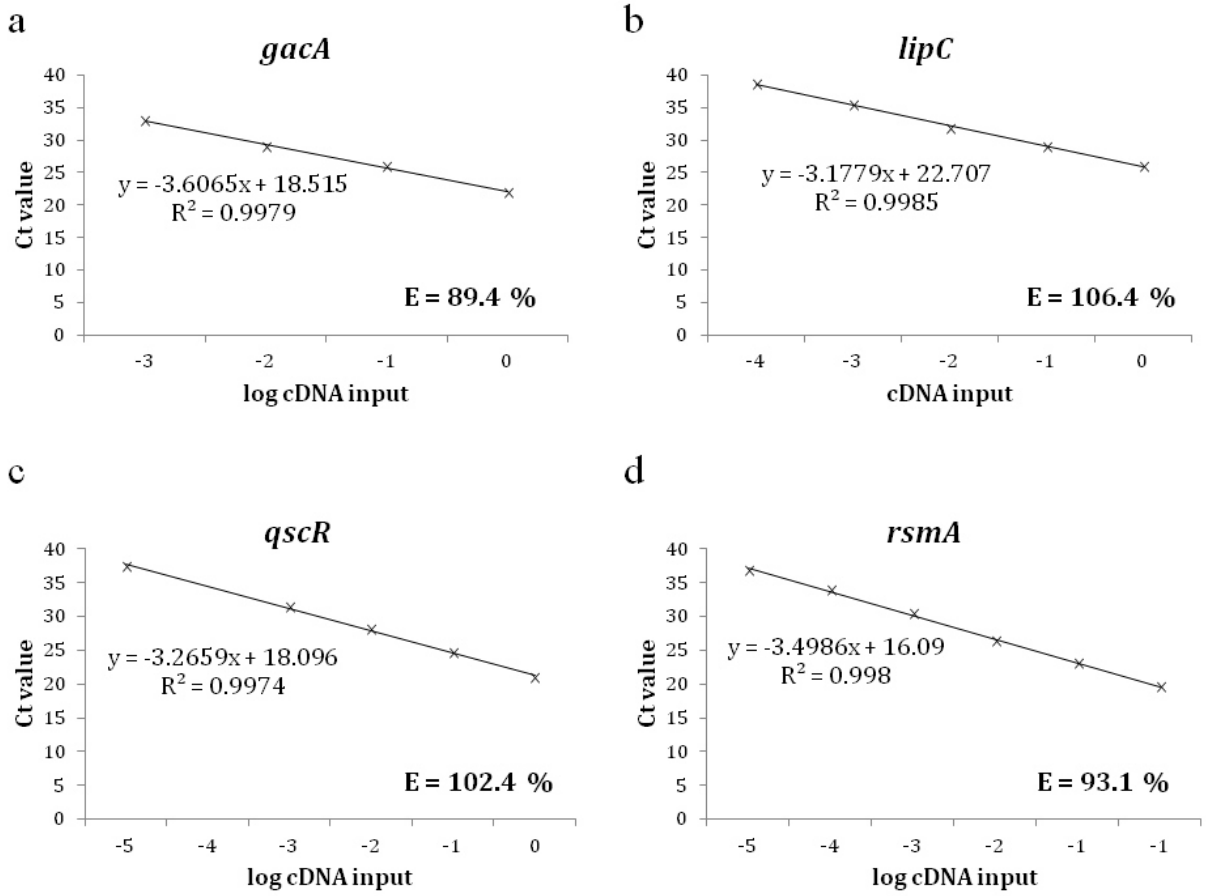

Fig. S5 PCR efficiency of the genes for regulatory factors GacA, LipC, QscR, and RsmA in *P. aeruginosa* PAO1. PCR efficiencies of *gacA* (a), *lipC* (b), *qscR* (c), and *rsmA* (d) were determined using the serial dilution method and standard curves were generated by plotting the cycle at the fluorescence threshold (C_t value) against the log of target DNA concentration. The efficiency in percent was calculated from the slope of the regression curve and is indicated by *E* in the graph

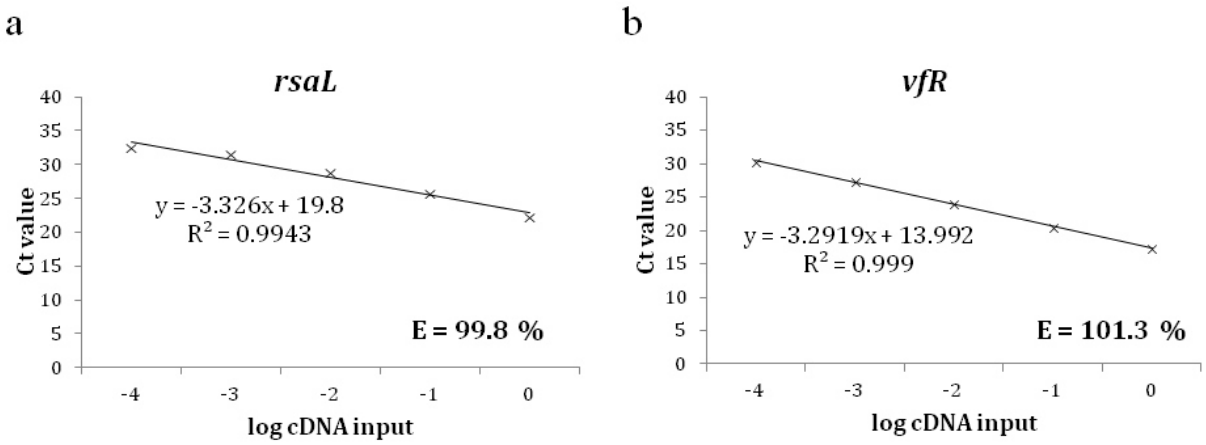

Fig. S6 PCR efficiency of the genes for regulatory factors RsaL and VfR in *P. aeruginosa* PAO1. PCR efficiencies of *rsaL* (a) and *vfR* (b) were determined using the serial dilution method and standard curves were generated by plotting the cycle at the fluorescence threshold (C_t value) against the log of target DNA concentration. The efficiency in percent was calculated from the slope of the regression curve and is indicated by *E* in the graph

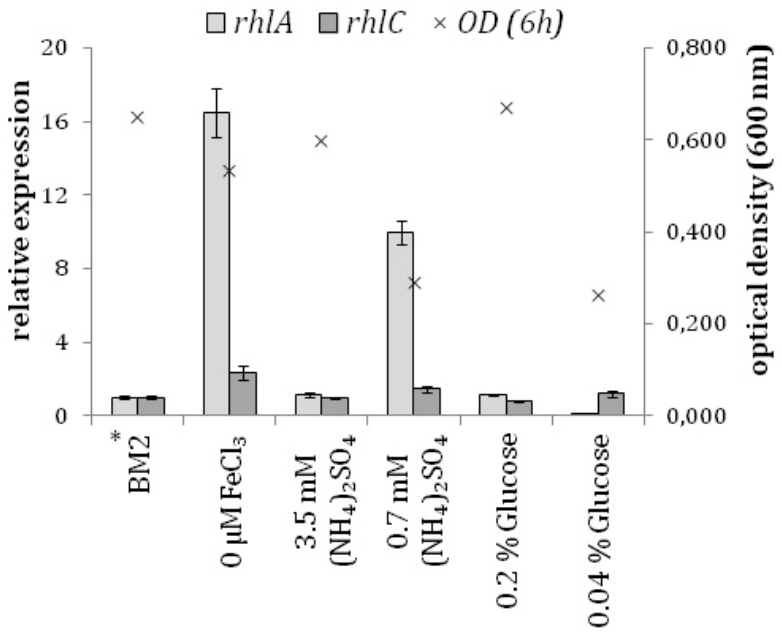

Fig. S7 Relative gene expression of rhamnosyltransferases 1 and 2 (*rhlA* and *rhlC,* respectively) in *P. aeruginosa* PAO1 in response to various nutrient offers. The gene expression of *rhlA* and *rhlC* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as bars during shake flask experiments with different compositions of standard BM2 medium. Samples were collected at mid-exponential phase after 6 hours of cultivations. Expression of genes in standard BM2 medium (10 µM $FeCl_3$; 7 mM $(NH_4)_2SO_4$; 0.4 % glucose) was used as a reference for the calculation of expression ratios and is indicated by the asterisk

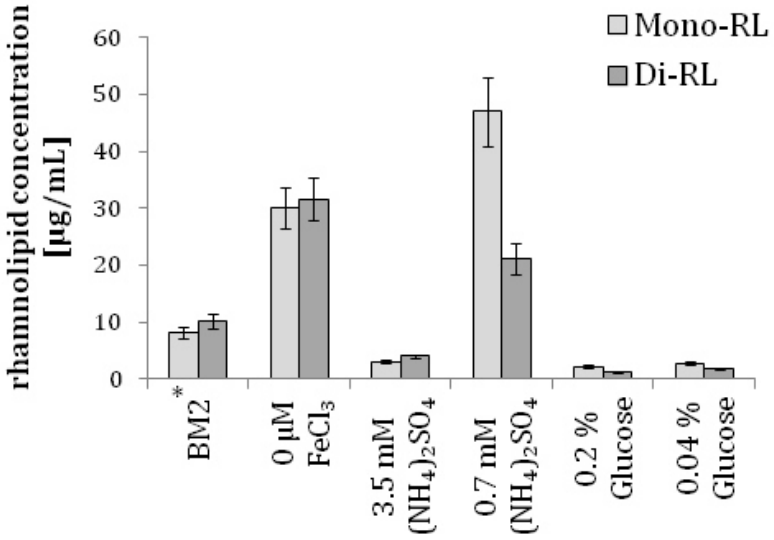

Fig. S8 Rhamnolipid production of *P. aeruginosa* PAO1 in response to various nutrient offers. Mono- and dirhamnolipids were quantified in the supernatant of shake flask cultures of *P. aeruginosa* PAO1 after 24 hours incubation under different concentrations of nitrogen, iron and carbon using LC/MS/MS. Standard BM2 medium is considered as a reference with 10 µM $FeCl_3$; 7 mM $(NH_4)_2SO_4$; 0.4 % glucose and indicated by the asterisk

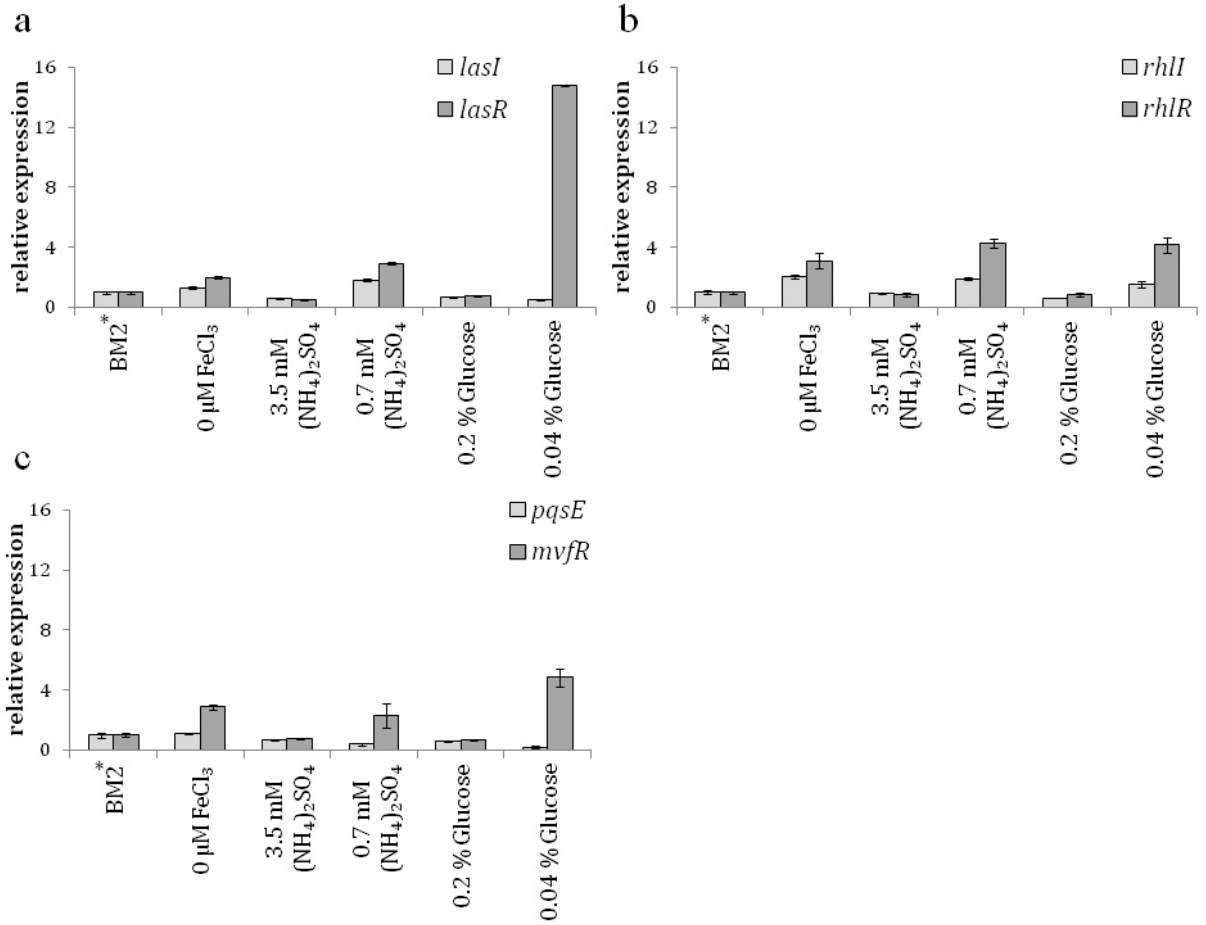

Fig. S9 Relative expression of the genes of the *las*, *rhl*, and *pqs* QS systems in *P. aeruginosa* PAO1 in response to various nutrient offers. The gene expression of *lasI, lasR, rhlI, rhlR, pqsE,* and *mvfR* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as bars during shake flask experiments with different compositions of standard BM2 medium. Samples were collected at mid-exponential phase after 6 hours of cultivations. Expression of genes in standard BM2 medium (10 µM $FeCl_3$; 7 mM $(NH_4)_2SO_4$; 0.4 % glucose) was used as a reference for the calculation of expression ratios and is indicated by the asterisk

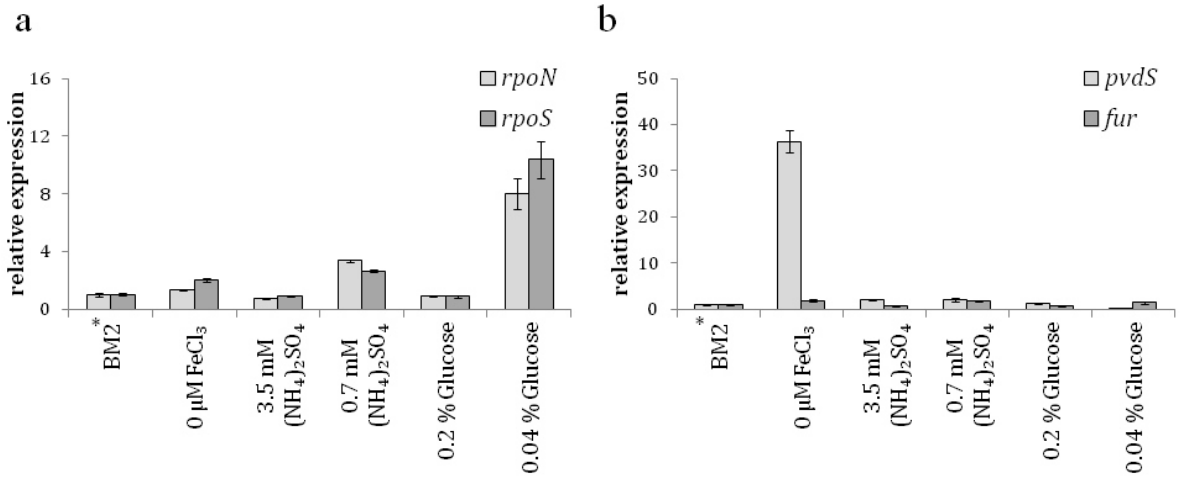

Fig. S10 Relative gene expression of sigma factors RpoN, RpoS, and PvdS in *P. aeruginosa* PAO1 in response to various nutrient offers. The gene expression of *rpoN, rpoS, pvdS,* and *fur* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as bars during shake flask experiments with different compositions of standard BM2 medium. Samples were then collected at mid-exponential phase after 6 hours of cultivations. Expression of genes in standard BM2 medium (10 µM $FeCl_3$; 7 mM $(NH_4)_2SO_4$; 0.4 % glucose) was used as a reference for the calculation of expression ratios and is indicated by the asterisk

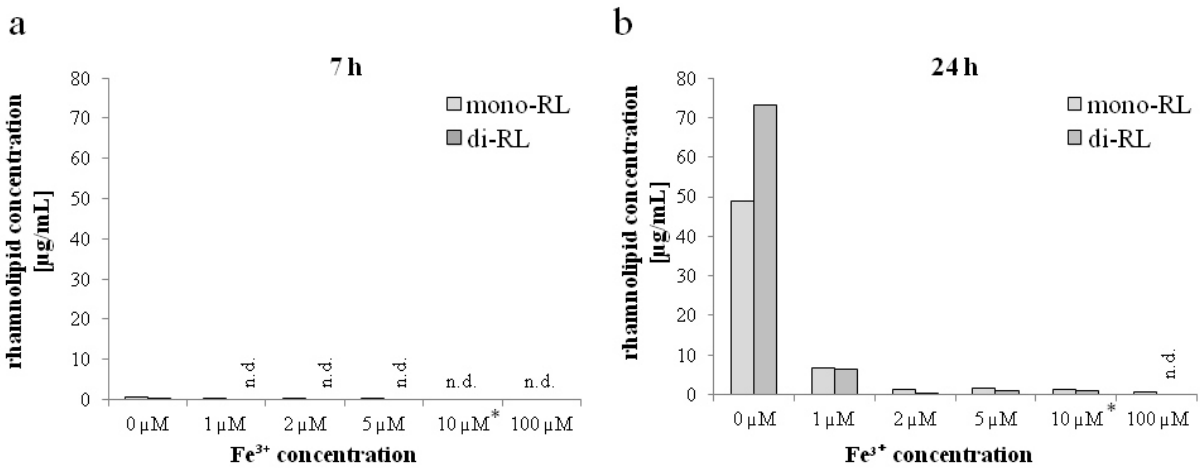

Fig S11 Rhamnolipid production of *P. aeruginosa* PAO1 in response to various concentrations of Fe^{3+}. Both mono- and dirhamnolipids were quantified in the supernatant of cultures of shake flask cultures of *P. aeruginosa* PAO1 grown under different concentrations of Fe^{3+} using LC/MS/MS

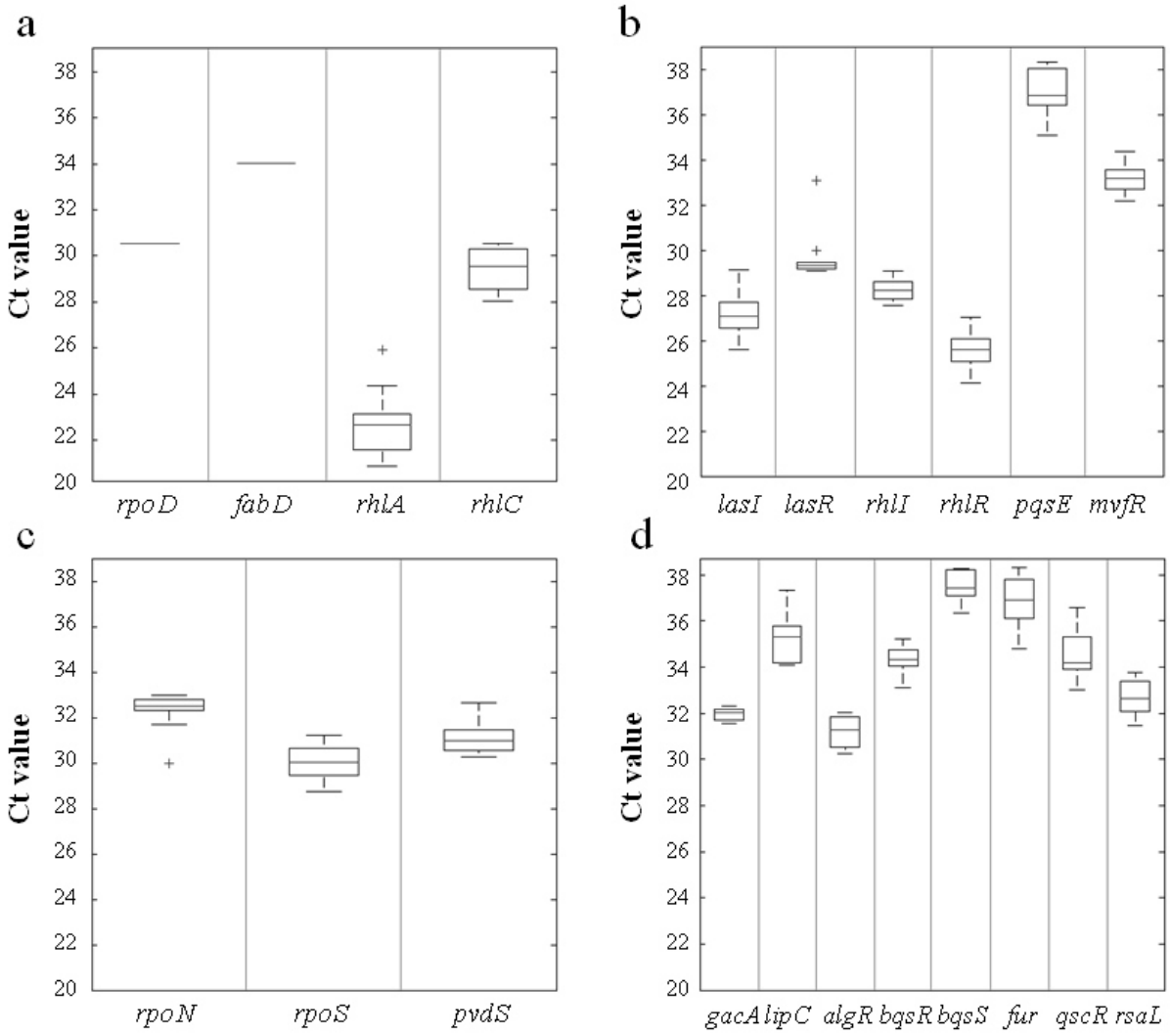

Fig S12 Ct values of reference genes and target genes. Each box represents all Ct values that were measured for one target gene over the entire time course of the cultivation and were later normalised against the two reference genes *rpoD* and *fabD*. Outliers are indicated by plus signs. As expression of the two reference genes is considered to be constant, they appear as horizontal lines in the box plot diagram. For the quantitative Real-time PCR, equal amounts of cDNA, that is 0.4 ng/ µL, were used for each sample and gene

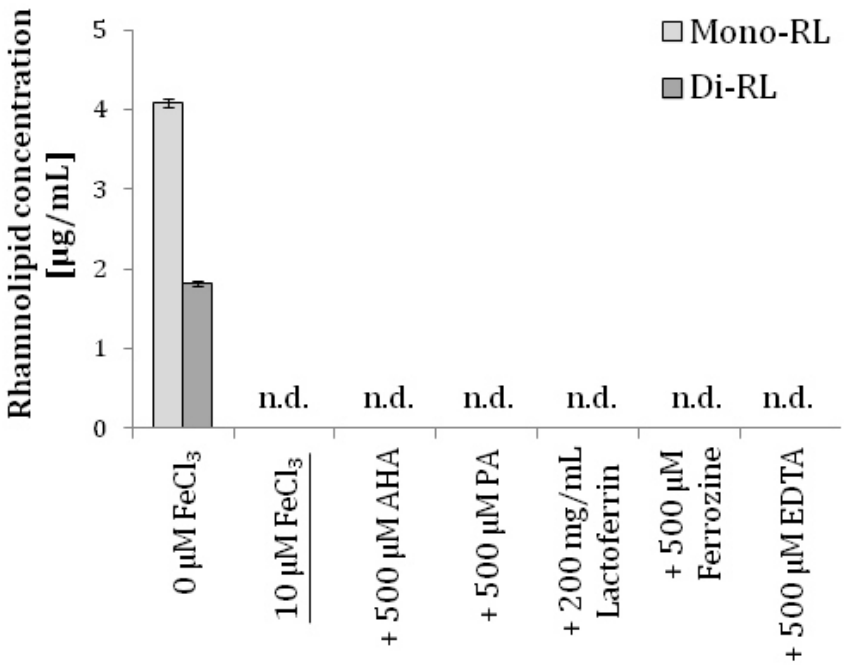

Fig. S13 Rhamnolipid production of *P. aeruginosa* PAO1 in response to iron chelating compounds. Both mono- and di-rhamnolipids were quantified in the supernatant of cultures of shaking flask cultures of *P. aeruginosa* PAO1 grown in BM2 medium supplemented with different iron chelators. Prior to incubation, medium was treated with a given chelator and samples were then collected at mid-exponential phase after 6 hours of cultivations. Rhamnolipid production in cultures with standard BM2 Medium containing 10 µM $FeCl_3$ was used as a reference and is therefore underlined

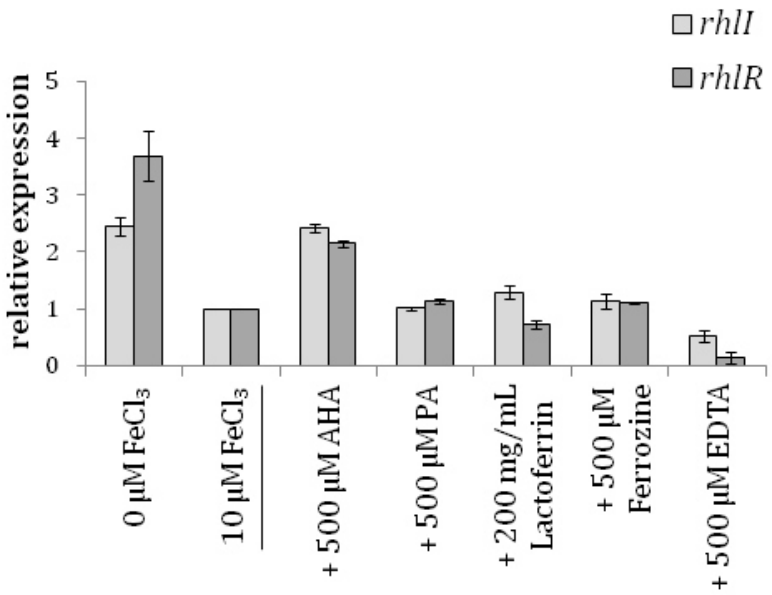

Fig. S14 Expression of the genes of the *rhl* QS systems in *Pseudomonas aeruginosa* PAO1 in response to iron chelating compounds. The gene expression of *rhlI* and *rhlR* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as bars with supplementation of different iron chelators during shake flask experiments. Prior to incubation, medium was treated with a given chelator and samples were then collected at mid-exponential phase after 6 hours of cultivations. Expression of genes in standard BM2 Medium with 10 µM $FeCl_3$ was used as a reference for the calculation of expression ratios and is therefore underlined

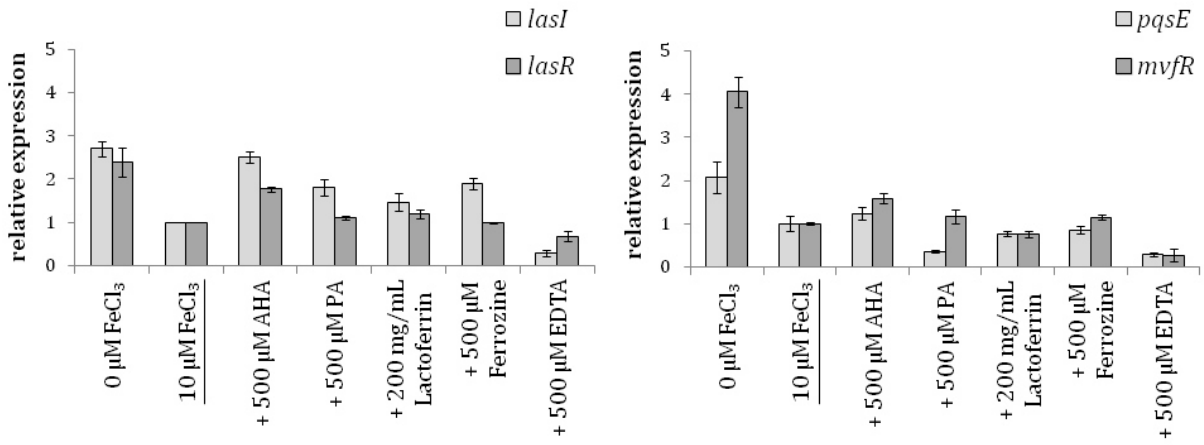

Fig. S15 Expression of the genes of the *las* and *pqs* QS systems in *Pseudomonas aeruginosa* PAO1 in response to iron chelating compounds. The gene expression of *lasI, lasR, pqsE* and *mvfR* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as bars with supplementation of different iron chelators during shake flask experiments. Prior to incubation, medium was treated with a given chelator and samples were then collected at mid-exponential phase after 6 hours of cultivations. Expression of genes in standard BM2 Medium with 10 µM $FeCl_3$ was used as a reference for the calculation of expression ratios and is therefore underlined

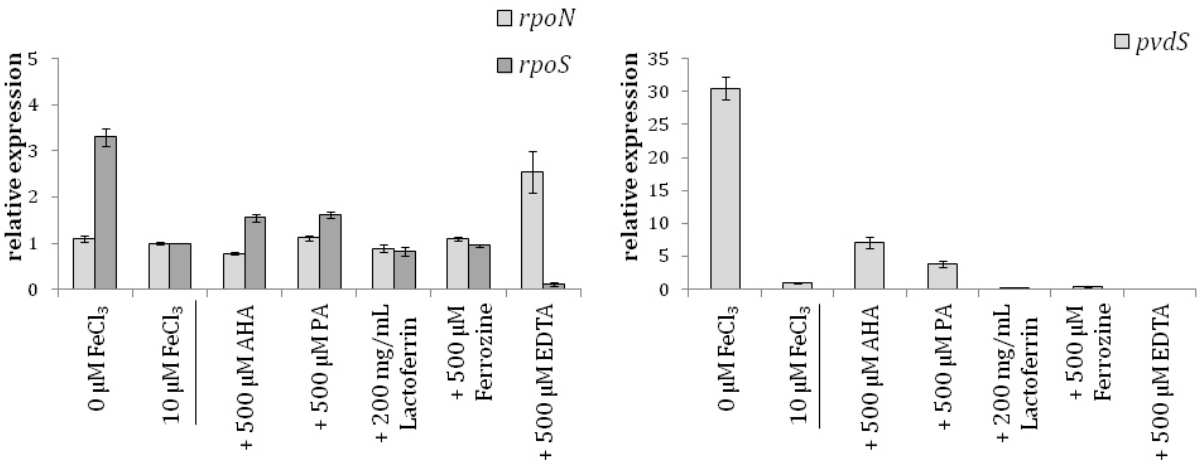

Fig. S16 Relative gene expression of sigma factors RpoN, RpoS, and PvdS in *Pseudomonas aeruginosa* PAO1 in response to iron chelating compounds. The gene expression of *rpoN, rpoS,* and *pvdS* was normalised to *rpoD* and *fabD* gene expression. The relative expressions are shown as bars with supplementation of different iron chelators during shake flask experiments. Prior to incubation, medium was treated with a given chelator and samples were then collected at mid-exponential phase after 6 hours of cultivations. Expression of genes in standard BM2 Medium with 10 µM $FeCl_3$ was used as a reference for the calculation of expression ratios and is therefore underlined

iii. Gene Repertoire

Name	**Locus Tag**	**Product Name**
algR	PA5261	Alginate Biosynthesis Regulatory Protein, AlgR
bqsR	PA2657	Probable Two-Component Response Regulator, BqsR
bqsS	PA2656	Probable Two-Component Sensor, BqsS
fabD	PA2968	Malonyl-CoA-[acyl-carrier-protein] transacylase, FabD
fur	PA4764	Ferric Uptake Regulation Protein, Fur
gacA	PA2586	Response Regulator, GacA
lasI	PA1432	Autoinducer Synthesis Protein, LasI
lasR	PA1430	Transcriptional Regulator, LasR
lipC	PA4813	Lipase, LipC
mvfR	PA1003	Transcriptional Regulator, MvfR (=pqsR)
pqsE	PA1000	Quinolone Signal Response Protein, PqsE
pvdS	PA2426	Sigma Factor for Iron Limitation, PvdS
qscR	PA1898	Quorum Sensing Control Repressor, QscR
rhlA	PA3479	Rhamnosyltransferase 1 Chain A, RhlA
rhlC	PA1130	Rhamnosyltransferase 2, RhlC
rhlI	PA3476	Autoinducer Synthesis Protein, RhlI
rhlR	PA3477	Transcriptional Regulator, RhlR
rpoD	PA0576	Sigma Factor, RpoD
rpoN	PA4462	Sigma Factor for Nitrogen Limitation, RpoN, σ^{54}
rpoS	PA3622	Stationary Phase Sigma Factor, RpoS
rsaL	PA1431	Regulatory Protein, RslA
rsmA	PA0905	Carbon Storage Regulator, RsmA
vfR	PA0652	Transcriptional Regulator, VfR

X. Curriculum Vitae

Personal Data

Name:	Anke Schmidberger
Date of Birth:	10.09.1985

Education

02/2011 – 02/2014	Doctoral Thesis at the Institute of Functional Interfaces of the Karlsruhe Institute of Technology *„Molecular Characterisation of Cultivation Processes - Stress-induced Rhamnolipid Production“*
12/2010	Diploma in Biology, Grade: Magna cum laude
02/2010 – 12/2010	Diploma Thesis in Mikael Jondals Group at the Centre of Microbiology, Tumour and Cell Biology, Karolinska Institutet, Stockholm, Sweden *“Tolerising or Activating CD8 positive transgenic T Cells against a tumour antigen using peptide treated immature or CpG activated Dendritic Cells”*
06/2009 – 08/2009	Project Work in Prof. Dr. Martin Eilers Group at the Chair of Physiological Chemistry II, University of Würzburg *"Optimisation of a protocol for a GST pulldown assay of Myc-Max-interaction in vitro"*
07/2008 – 09/2008	Project Work in Prof. Dr. Aladar Szalays Group, Chair of Biochemistry, University of Würzburg *“Treatment of Human Prostate Tumour Xenografts in Nude mice with microorganisms”*

10/2006	Course of Biology at the University of Würzburg Major: Microbiology (Virology/ Immunology) Minors: Genetics, Physiological Chemistry
10/2004 – 09/2006	Course of Chemistry at the Julius-Maximilians-University of Würzburg
07/2004	Abitur, Robert-Mayer-Gymnasium, Heilbronn

Publications

2013 **A Schmidberger**, M Henkel, R Hausmann and T Schwartz
„Expression of genes involved in rhamnolipid synthesis in *Pseudomonas aeruginosa* PAO1 in a bioreactor cultivation", Journal of Applied Microbiology and Biotechnology; *(DOI 10.1007/s00253-013-4891-0)*

2013 M Henkel, **A Schmidberger** , C Kühnert, J Beuker, T Bernard, T Schwartz, C Syldatk and R Hausmann
„Kinetic modeling of the time course of N-butyryl-homoserine lactone concentration during batch cultivations of *Pseudomonas aeruginosa* PAO1", Journal of Applied Microbiology and Biotechnology; *(DOI 10.1007/s00253-013-5024-5)*

2014 **A Schmidberger**, M Henkel, R Hausmann and T Schwartz
Influence of Ferric Iron on Gene Expression and Rhamnolipid Synthesis during Batch Cultivation of *Pseudomonas aeruginosa* PAO1", Journal of Applied Microbiology and Biotechnology; *(DOI* 10.1007/s00253-014-5747-y)

2014 M Henkel, **A Schmidberger**, M Vogelbacher, C Kühnert, J Beuker, T Bernard, T Schwartz, C Syldatk, and R Hausmann
"Kinetic Modeling of Rhamnolipid Production of *Pseudomonas aeruginosa* PAO1 including Cell-density Dependent Regulation", Journal of Applied Microbiology and Biotechnology; *(DOI* 10.1007/s00253-014-5750-3)

Talks

03/2013 „Iron-dependent Regulation of Rhamnolipid Synthesis in *Pseudomonas aeruginosa* PAO1"; Annual Conference of the Association for General and Applied Microbiology (VAAM), Bremen

Knowledge of Foreign Languages/ International Experience

German: native
English: fluent
Swedish: fluent
French: basics

02/2010 – 12/2010	Graduand at the Karolinska Institutet, Stockholm, Sweden
01/2008 – 06/2008	ERASMUS Student at the Umeå Universitet. Umeå, Sweden Relevant Courses: Tumour Biology, Immunology
08/2001 – 07/2002	High School Year, Toledo, Ohio, USA

Internships/ Research Assistencies

07/2009 – 12/2009	Research Assistant at the Chair of Biochemistry at the University of Würzburg
03/2009	4th International Symposium 2009 of the Graduate School of Life Sciences' der Universität Würzburg
10/2008 – 02/2009	Research Assistant at the Chair of Hygiene and Microbiology at the University Clinic of Würzburg

Software Skills

MS-Office (Word, PowerPoint, Excel), BioGazelle QbasePlus, Matlab, Simulink, Stateflow

Advanced Training

10/2012	Introductory Course "Matlab, Simulink and Stateflow", ITK Engineering AG, Karlsruhe
12/2011	2-day qPCR course, Biogazelle, Gent