Carbon source driven virulence factors generation by *Pseudomonas aeruginosa*, implications for application in bioelectrochemical systems

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“But thou, O Daniel, shut up the words, and seal the book, even to the time of the end: many shall run to and fro, and knowledge shall be increased.” (Daniel 12:4 KJV)
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# Table of Contents

Acknowledgements ......................................................................................................................... iii
Table of Contents ............................................................................................................................. iv
List of Tables ...................................................................................................................................... viii
List of Figures ..................................................................................................................................... ix
Abbreviations and acronyms ......................................................................................................... xiii
Abstract .......................................................................................................................................... xiv
Zusammenfassung ........................................................................................................................... xv

## Chapter One

Background ........................................................................................................................................ 1

1.1 Introduction ................................................................................................................................... 1

1.2 From QS to Virulence ................................................................................................................... 3

1.2.1 Interplay between QS and two component signalling system ........................................... 6

1.3 Carbon source mediated synergistic virulence factors generation ......................................... 8

1.4 *P. aeruginosa* virulence factors applicable for current generation ........................................ 11

1.5 The BES technology .................................................................................................................. 16

1.5.1 Methodology in BES .......................................................................................................... 18

1.5.2 Electron transfer ................................................................................................................ 19

1.6 Hypothesis and aim of the study ............................................................................................ 21

## Chapter Two

Materials and methods ..................................................................................................................... 24

2.1 *P. aeruginosa* strains ................................................................................................................ 24

2.2 Media and culture conditions .................................................................................................. 24

2.2.1 *Pseudomonas* Agar ......................................................................................................... 25

2.2.2 Motility assay agar .......................................................................................................... 25

2.2.3 Synthetic cystic fibrosis medium .................................................................................... 25

2.3 Shake flask experiments ......................................................................................................... 26

2.4 Biomass measurements ........................................................................................................... 26

2.5 Molecular procedures ............................................................................................................ 27
2.5.1 PCR and Gibson assembly ................................................................. 27
2.5.2 Transformation ................................................................. 27
2.5.3 Mutant generation using homologous recombination ............... 28
2.6 BES setup and electrochemical procedures .................................. 30
2.7 Phenazine utilization and production analysis ................................ 33
2.8 Analytical procedures ................................................................. 34
   2.8.1 Detection and quantification of phenazines ......................... 34
   2.8.2 Detection and quantification of carbon sources and metabolites 35
   2.8.3 AHL detection and quantification ............................................. 35

Chapter Three
Strain and substrate dependent redox mediator and electricity production by Pseudomonas aeruginosa ................................................................. 37
   3.1 Background ........................................................................ 37
   3.2 Experimental set up ................................................................. 39
   3.3 Results ............................................................................. 40
      3.3.1 Single substrate metabolism ............................................ 40
      3.3.2 Single substrate phenazine and current generation ............ 43
      3.3.3 Co-substrate consumption and bio-electrochemical utilisation 46
      3.3.4 Cyclic voltammetry analysis of redox activity .................... 48
      3.3.5 Evaluating differences in usage and production of phenazines 51
   3.4 Discussion ........................................................................ 54
      3.4.1 Substrate preference of P. aeruginosa strains .................... 54
      3.4.2 Strains dictate redox mediator and electric current production 56
      3.4.3 PCA mainly responsible for reversible redox cycling ............. 58
      3.4.4 Anodic electron discharge is a metabolic side reaction under oxygen limitation 59
   3.5 Conclusions ...................................................................... 61

Chapter Four
Electrochemical potential influences phenazine production and electron transfer and consequently electric current generation ......................................................... 62
4.1 Background ........................................................................................................................................................................62
4.2 Experimental set-up ..................................................................................................................................................................64
4.3 Results ........................................................................................................................................................................................................64
  4.3.1 Phenazine production and current generation at cathodic potentials ..............................64
  4.3.2 Phenazine production and current generation at anodic potentials .................................66
  4.3.3 Growth and carbon source uptake ..................................................................................73
  4.3.4 Overall influence of the applied electrode potential on P. aeruginosa physiology ......77
4.4 Discussion ..................................................................................................................................................................................................79
  4.4.1 Increased electroactivity is a function of an appropriate potential .................................79
4.5 Conclusion ................................................................................................................................................................................................82

Chapter Five
Quorum sensing activation and virulence factors production by P. aeruginosa in different nutritional backgrounds ........................................................................................................................................................................................................83
  5.1 Background ........................................................................................................................................................................83
  5.2 Experimental set up ................................................................................................................................................................84
  5.3 Results ................................................................................................................................................................................................86
    5.3.1 Growth and virulence generation in minimal medium ...................................................86
  5.4 Growth and virulence generation in nutrient-rich conditions .............................................110
  5.5 Discussion .....................................................................................................................................................................................................112
    5.5.1 Substrate consumption and biomass generation ...........................................................112
    5.5.2 The carbon source determines the level of virulence generation in minimal medium .....................................................................................................................................................................................................114
    5.5.3 Substrate dependent AHL QS signal concentration .....................................................117
    5.5.4 Fermentation product 2,3-BD influences production of the virulence factor phenazine by strain PA14 in host environment ..........................................................................................................................................................................................118
  5.6 Conclusions .........................................................................................................................................................................................................................................120

Chapter Six
The role of 2,3-BD in signalling and virulence stimulation in Pseudomonas aeruginosa PA14 .121
  6.1 Background ...........................................................................................................................................................................121
Chapter Seven
The two-component systems RcsC/RcsB and PvrS/PvrR are involved in the perception and regulation of 2,3 BD-stimulated virulence factors production ................................................................. 137

7.1 Experimental set up ........................................................................................................... 138
7.2 Results ............................................................................................................................... 139

7.2.1 Signalling of volatile compounds via the plant ethylene and cytokinin systems: are there similarities with P. aeruginosa? ........................................................................................................... 139

7.3 Discussion .......................................................................................................................... 146

7.3.1 The two-component systems RcsC/RcsB and PvrS/PvrR in the PAPI virulence island are involved in the regulation of phenazine production .............................................. 146

7.4 Conclusion ........................................................................................................................ 148

Chapter Eight
Summary, conclusions and outlook ........................................................................................ 149

9 Appendices .......................................................................................................................... 153

9.1 Appendix 1: Phenazine production time profiles for Chapter 5-section 5.3.1.3. .......... 153
9.2 Appendix 2: Primers used in the study ........................................................................... 155
9.3 Appendix 3: Motility assay (Chapter 5-section 5.3.1.4.1) ............................................... 158
9.4 Appendix 4: PCA-like compound produced by RcsC/RcsB and PvrS/PvrR mutant with glucose (Chapter 6-section 7.2.1.2) ..................................................................................... 162

References ............................................................................................................................ 163
List of Tables

Table 3.1: Consumption rates of the three carbon substrates ........................................................ 40
Table 3.2: Coulombic efficiencies of the conversion of different substrates to electric current... 45
Table 3.3: Final bioreactor biomass as cell dry weight and biofilm tendency for different carbon sources ............................................................................................................. 45
Table 3.4: Electrochemical and chromatographic phenazine analysis for strain PA14 ............ 48
Table 3.5: Electrochemical and chromatographic phenazine analysis for strain KRP1 and PAO1 ........................................................................................................................................ 49
Table 3.6: DNA sequence identity (in %) of the phenazine genes in a pair-wise comparison of the three P. aeruginosa strains. The number in the bracket indicates the number of nucleotide miss-matches ............................................................................ 53
Table 4.1: Formal potentials (E_{1/2}) of the two peak systems observed at cathodic potentials, phenazine concentration and pH. ................................................................................................. 73
Table 4.2: Growth and biofilm formation at the different potentials ........................................ 76
Table 4.3: Influence of applied potential on the physiology of P. aeruginosa. ............................. 77
Table 5.1: Maximum phenazines production by strain PA14 and strain KRP1 normalized per biomass ................................................................................................................................................ 96
Table 5.2: Maximum phenazines production by strains PA14 and strain KRP1 normalized per biomass for the additional substrates succinate and octanoate. ........................................ 99
Table 5.3: Distance covered from the point of inoculation by strain PA14 during swimming, swarming and twitching ........................................................................................................... 105
Table 5.4: Distance covered from the point of inoculation by strain KRP1 during swimming, swarming and twitching .......................................................................................................... 105
Table 7.1: Similarities of P. aeruginosa two component systems with CRE1. ......................... 141
Table 9.1: Primers used in the sequencing of phenazine operons (Chapter 3). ......................... 155
Table 9.2: Primers used in the generation and confirmation of the butanoate and 2,3-BD oxidation pathway mutants (Chapter 7) .................................................................................. 156
Table 9.3: Primers for the generation of the two-component system mutants in the PAPI Island ............................................................................................................................................. 157
List of Figures

Figure 1.1: Intricate interconnection of the AHL and Quinolone QS systems of *P. aeruginosa* (modified from (19, 31)).................................................................................................................. 5

Figure 1.2: Overview of the biosynthesis of the common phenazines produced by *P. aeruginosa*........................................................................................................................... 14

Figure 1.3: The general working principle of MFC and a potentiostatically controlled BES. ..... 18

Figure 1.4: Schematic representation of (a) direct electron transfer and (b) mediated electron transfer (adopted from Schroeder (2007)(71)). ........................................................... 20

Figure 2.1: a) pEMG deletion vector for introduction of flanking regions into the genome and b) PSW-1 for creating strand breaks on the I-SceI sites (adopted from (92))............ 29

Figure 2.2: Introduction of the flanking regions (FR1 and FR2) of the gene of interest into the genome using the pMEG plasmid ...................................................................................... 29

Figure 2.3: Generation of double strand breaks and homologous recombination......................... 31

Figure 2.4: BES set up showing the reactor and the carbon electrodes used. .............................. 32

Figure 2.5: Cyclic voltamograms of PCA (30 µg mL-1), PYO (15 µg mL-1), and a mixture of PCA and PYO (at the same concentration (left panel) and 1-HP (15 µg mL-1) and PCN (15 µg mL-1 (right panel) at pH 6.9. ........................................................... 32

Figure 3.1: Experimental approach for the comparison of the substrate dependent current and redox mediator production by the *P. aeruginosa* strains in BES. .................. 40

Figure 3.2: Carbon substrate consumption (a, b, c) and metabolite formation time profiles (d, e, f) of three *P. aeruginosa* strains in oxygen-limited bioelectrochemical experiments; with *P. aeruginosa* strain PA14 on top, *Pseudomonas* strain KRP1 in the middle, and *P. aeruginosa* strain PAO1 at the bottom................................. 42

Figure 3.3: Current generation and phenazines production by *P. aeruginosa* PA14 (top), KRP1 (middle) and PAO1 (bottom) grown with the substrates glucose (left), 2,3-BD (vertical middle), and ethanol (right)......................................................................... 44

Figure 3.4: Carbon substrate uptake of cultures supplied with equimolar glucose/2,3-BD (a, b, c) and related electric current generation and phenazine concentrations (d, e, f): strain PA14 (top); strain KRP1 (middle) and strain PAO1 (bottom). ............... 47

Figure 3.5: Cyclic voltamograms of the three *P. aeruginosa* strains (vertical variable) grown with the three carbon sources (horizontal variable). ......................................................... 50
Figure 3.6: Phenazine utilization assay with defined phenazine concentrations. ............... 52
Figure 3.7: Analysis of transcription regulatory elements of the phenazine gene clusters. .... 54
Figure 3.8: Observed maximum current densities for *P. aeruginosa* strains PA14, KRP1 and PAO1 grown in the presence of glucose, 2,3-BD, ethanol or equimolar glucose/2,3-BD; averaged from three independent biological replicates; except KRP1:2,3-BD in duplicates. b) shows a photograph of the used bioelectrochemical setup. ........................................... 55
Figure 4.1: Current generation and redox mediator production at cathodic potentials. ........ 65
Figure 4.2: Current generation at anodic potentials .......................................................... 67
Figure 4.3: Phenazine and pyoverdine production at anodic potentials ....................... 69
Figure 4.4: Redox peak systems 1 and 2 (PS 1 and PS 2) ............................................... 70
Figure 4.5: Cyclic voltamograms for all cultures .............................................................. 71
Figure 4.6: A comparison of the cultures (with cells) and supernatants of the same cultures using clean electrodes ................................................................. 73
Figure 4.7: Carbon source uptake at different electrode potentials and metabolite production over time. Notice that the carbon source is plotted on the left Y axis and the metabolites ketogluconate, gluconate, acetoin and acetate are plotted on the right Y axis ................................................................. 75
Figure 4.8: Biofilm formation at the air-liquid interface and around the electrodes ......... 76
Figure 5.1: Schematic representation of the experimental design for cultivation of *P. aeruginosa* strains PA14 and KRP1 in media containing different substrates ............... 85
Figure 5.2: Glucose (a, b) and 2,3-BD (c) utilization during growth under aerobic (A) and micro-aerobic (M) conditions ................................................................. 87
Figure 5.3: Calculated carbon source uptake rates of glucose and 2,3-BD ....................... 88
Figure 5.4: Carbon source utilisation in a co-feed of equimolar glucose/2,3 BD mixture, and the metabolites produced under aerobic (A) and microaerobic (M) conditions for strains: (a, b) PA14 and (b, c) KRP1 ......................................................... 89
Figure 5.5: Growth curves of *P. aeruginosa* strains PA14 and KRP1 in medium containing glucose and 2,3-BD ................................................................. 90
Figure 5.6: Succinate (left) and octanoate (right) consumption over time by strain ......... 92
Figure 5.7: Calculated carbon source uptake rates of octanoate and succinate .......... 92
Figure 5.8: Growth curves of *P. aeruginosa* strains PA14 and KRP1 in medium containing (left) succinate and (right) octanoate..........................93

Figure 5.9: Calculated growth rates in medium supplied with succinate and octanoate............94

Figure 5.10: Pyoverdine production by strains PA14 and KRP1 in medium containing (a) glucose, (b) 2,3-BD and (c) equimolar mix of glucose/2,3-BD.................................97

Figure 5.11: Rhamnolipid production in minimal medium provided with (a) glucose, (b) 2,3-BD and (c) glucose/2,3-BD co-feed.........................................................98

Figure 5.12: Pyoverdine production by strains PA14 and KRP1 in medium containing (Left) succinate and (Right) octanoate .........................................................101

Figure 5.13: Rhamnolipid production in minimal medium provided with (a) succinate, (b) octanoate........................................................................................................101

Figure 5.14: Percentage of biomass and virulence factors production in comparison to the highest measured of the specific parameters.................................103

Figure 5.15: C-4-HSL production by cultures provided with different carbon sources.........108

Figure 5.16: 3-oxododecanoylhomoserine lactone production by cultures provided with different carbon sources.................................................................109

Figure 5.17: Growth, phenazine and pyoverdine production in SCFM medium with, additional, glucose or 2,3-BD.................................................................112

Figure 6.1: Schematic representation of the *P. aeruginosa* butanoate pathway. .................124

Figure 6.2: Schematic representation of the 2,3-butanediol catabolism pathway....................124

Figure 6.3: Growth of acetolactate mutants in AB medium containing glucose and 2,3-BD....126

Figure 6.4: Carbon source uptake and metabolites graphs for the wild type and acetolactate synthase mutants.................................................................127

Figure 6.5: Growth and pH change of the wild type and acetolactate synthase mutant in MOPS buffered medium.................................................................128

Figure 6.6: Carbon source uptake and metabolites produced by the wild type and acetolactate synthase mutants grown in MOPS buffered medium.................129

Figure 6.7: Growth and carbons source uptake of the 2,3-BD catabolic and acetolactate synthase mutants in AB medium..........................................................130

Figure 6.8: Phenazine and pyoverdine production by (left) the wild type and (right) butanoate pathway mutant with 2,3-BD in AB medium.................................132
Figure 6.9: Phenazines and pyoverdine production by the wild type and butanoate pathway mutant grown in MOPS medium provided with glucose (also see Figure 6.5)...... 133
Figure 6.10: Maximum phenazine and pyoverdine concentration corrected per OD. ............... 133
Figure 7.1: Growth of the Rcs and Pvr two-component system mutant provided with different substrates................................................................. 143
Figure 7.2: Phenazine and pyoverdine production by the RcsC/RcsB and PvrS/PvrR two-component system mutant (for comparison with the wild type also see Figure 9.1 and Figure 9.2). ................................................................. 145
Figure 9.1: Phenazine production over timer in medium containing glucose, 2,3-BD and a mixture of glucose/2,3-BD (Chapter 4-section 5.3.1.3). ................................. 153
Figure 9.2: Phenazine production over time by cultures provided with (top) succinate and (bottom) octanoate (Chapter 5-section 5.3.1.3)................................................... 154
Figure 9.3: Swimming, swarming and twitching with glucose and 2,3-BD for strain PA14. .... 158
Figure 9.4: Swimming, swarming and twitching with equimolar glucose/2,3-BD for strain PA14. ........................................................................................................ 158
Figure 9.5: Swimming, swarming and twitching succinate and octanoate for strain PA14. ..... 159
Figure 9.6: Swimming, swarming and twitching with glucose and 2,3-BD for strain KRP1..... 160
Figure 9.7: Swimming, and twitching with equimolar glucose/2,3-BD for strain KRP1........... 160
Figure 9.8: Swimming, swarming and twitching succinate and octanoate for strain KRP1...... 161
Figure 9.9: a) Chromatogram showing PCA and the PCA-like molecule at 17.5 and 19.0 minutes respectively and b) the spectrum of the unknown compound................. 162
### Abbreviations and acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1-HP</td>
<td>1-hydroxyphenazine</td>
</tr>
<tr>
<td>2,3-BD</td>
<td>2,3-butanediol</td>
</tr>
<tr>
<td>AHL</td>
<td>acyl homoserine lactone</td>
</tr>
<tr>
<td>BES</td>
<td>bioelectrochemical systems</td>
</tr>
<tr>
<td>CDW</td>
<td>cell dry weight</td>
</tr>
<tr>
<td>CV</td>
<td>cyclic voltammetry</td>
</tr>
<tr>
<td>E&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>formal potential</td>
</tr>
<tr>
<td>GAC</td>
<td>global activator of antibiotic and cyanide synthesis</td>
</tr>
<tr>
<td>HHQ</td>
<td>2-heptyl-4-quinolone</td>
</tr>
<tr>
<td>HSL</td>
<td>homoserine lactone</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>MFC</td>
<td>microbial fuel cell</td>
</tr>
<tr>
<td>PCA</td>
<td>phenazine-1-carboxylic acid</td>
</tr>
<tr>
<td>PQS</td>
<td><em>Pseudomonas</em> quinolone signal</td>
</tr>
<tr>
<td>PYO</td>
<td>pyocyanin</td>
</tr>
<tr>
<td>Pyv</td>
<td>pyoverdine</td>
</tr>
<tr>
<td>QS</td>
<td>quorum sensing</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SHE</td>
<td>standard hydrogen electrode</td>
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Abstract

*Pseudomonas aeruginosa*, the ubiquitous quintessential pathogen, produces a wide array of virulence factors to propagate pathogenicity. In polymicrobial infections and competitive environmental niches, a concerted action of the virulence factors ensures its competitiveness and often dominance. The quorum sensing system controls the genes coding for these virulence factors ensuring a timely production to avoid unnecessary virulence activation. *P. aeruginosa* virulence factors, especially phenazines, have gained attention for biotechnological application, such as bioremediation or in bioelectrochemical systems (BES). For the latter, especially phenazine redox mediators and other pathogenicity factors like rhamnolipids and siderophores might play an important role in enabling biofilm formation on the electrodes. In mixed culture BES, bacteria metabolise substrates and liberated electrons are shuttled to an external electron acceptor (anode) via soluble redox mediators among other modes of electron transfer. *P. aeruginosa* plays a key role in these processes and displays tremendous metabolic versatility and nutrition influences its virulence generation. Like in natural communities, microorganisms often build synergistically interacting microbial consortia in wastewater BES. In these interactions, consumption of fermentation products from fermenters by *P. aeruginosa* leads to increased phenazine mediator production and consequently increased electroactivity of the whole community. Understanding the signalling mechanisms behind this natural phenomenon may allow the design of co-cultures for increased current production in BES or engineering phenazine redox mediator-based BES biocatalysts. Also, a detailed understanding of how the phenazines are employed under varying ecological conditions across the *P. aeruginosa* strains and their capacity as redox mediators in BES is yet to be fully achieved. This thesis first explored the applicability of the *P. aeruginosa* strains (PA14, KRP1 and PAO1) as redox mediator producers in BES. Next, the influence of one of the important BES parameters—electrochemical potential on the phenazine physiology and electric current generation of strain PA14 was examined. To further decipher how fermentation products influence the virulence generation, 2,3-butanediol (2,3-BD) metabolism pathway mutants were generated and their virulence generation assessed. Finally, the two-component systems involved in the perception of 2,3-BD were identified. Remarkable differences among the three strains in the production of phenazines, rhamnolipids and the tendency to form biofilms under the BES conditions and different substrates were revealed. Comparing all strains and carbon sources, the BES isolate KRP1 was the most electroactive when supplied with the three carbon sources considered. Phenazine-1-carboxylic acid (PCA) was found to play a major role in redox cycling under the BES conditions. The production and use of PCA in electron shuttling depended on the applied potential as well as the substrate. Overall the electrochemical potential impacts the electron transfer, growth and biofilm formation. These findings on the influence of the applied potential will enable appropriate poising of the electrode to harness the redox cycling potential of the phenazines. The RcsC/RcsB and PvrS/PvrR two-component systems were identified as, most likely, being responsible for the perception and regulation of the of 2,3-BD-stimulated virulence factors production; mutants lacking these systems exhibited reduced production of phenazines. These results provide fundamental insights that will lead to a more thorough understanding of carbon-source based virulence factor regulation and can be useful in designing synergistically interacting co-cultures, where *P. aeruginosa* is the redox mediator producer.
**Zusammenfassung**

Chapter One

Background

1.1 Introduction

The opportunistic human pathogen *Pseudomonas aeruginosa* exhibits a tremendous nutritional, environmental and host versatility (1). *P. aeruginosa* is a gram negative bacterium that was first described in 1872 (2). It has been isolated from diverse environments ranging from terrestrial and fresh water to open oceans (1, 3). It is also a common plant and animal pathogen. Its metabolic versatility ranges from the ability to grow on a wide range of organic substrates to degrading oil wastes (4).

It has been adversely mentioned as the most common opportunistic pathogen that causes nosocomial infections in cystic fibrosis and immunocompromised patients (5, 6). Its fitness in colonising and thriving in different environmental and host niches, and its capacity to generate antibiotic resistance reconciles with its resilience and difficulty to treat or eradicate (7, 8). The unique genome architecture of *P. aeruginosa*, containing what Mathee *et al*. (2007) refers to as “regions of genome plasticity”, confers it the ability to generate tools for niche adaptation. Through continuous evolution of these regions and acquisition of niche essential genes, *P. aeruginosa* diversifies its nutrient and host niches (9). With these outstanding capabilities, *P. aeruginosa* is undoubtedly one of the quintessential opportunistic pathogen, and has received immense attention in clinical and environmental research.

To propagate pathogenesis, *P. aeruginosa* employs several cell associated and intracellularly produced virulence factors; Type IV pili, adhesins and flagella for motility and adhesion to the
epithelium, Exotoxin A for protein synthesis inhibition and eventual cell death, exoenzyme S that damages lungs to aid dissemination, haemolysins (phospholipase C and rhamnolipids), which mediate the degradation of surfactants and loss of ciliary function, proteases which degrade elastin, immunoglobulins and compliment, and pyocyanin (PYO), which interferes with the physiology of the host cells (10-12). The cell density depended quorum sensing (QS) system controls the expression of these virulence factors, with reference to the prevailing environmental conditions to ensure timely economical production, and that the production coincides with appropriate thresholds. This timely production avoids unnecessary activation of the immune system (13).

Concerted action of the virulence factors also enables \textit{P. aeruginosa} to compete and interact in different microbial niches. In polymicrobial infections, besides the phenazines being directed at the host, they also act as antimicrobials, and QS signals might be used for interspecies co-operation (14). On the other hand, these antimicrobials as well as other virulence factors might be beneficial to other microorganisms in not only host conditions but also environmental niches. For instance, \textit{Burkholderia multivorans} was shown to use elastase from \textit{P. aeruginosa} and PYO can be utilised across different species for electron shuttling (15, 16). This capability of electron shuttling also has a promising potential for application in bioelectrochemical systems (BES) (17).

In polymicrobial infections and environmental consortia, besides competition interactions, microorganisms engage in synergistic or syntrophic interactions. For instance, it has been reported that \textit{P. aeruginosa} phenazines can confer electroactivity to an otherwise weakly electroactive community (18). Fermenters in return reduce the complex organic matter and carbon sources into fermentation products, which are utilised by \textit{Pseudomonas} and other
community members. This natural phenomenon can be utilised in the BES technology to design mixed cultures that can effectively transfer electrons to an external electron acceptor.

The following introduction explores the connection between QS and virulence generation. It provides an overview of the interaction of QS with environmental factors, with special focus on nutritional cues, and subsequent activation and generation of virulent factors. Lastly, the potential application of the virulence factors in the BES technology is discussed.

1.2 From QS to Virulence

The *P. aeruginosa* QS system is undoubtedly one of the most widely studied cell density signalling systems. *P. aeruginosa* uses this system to judiciously make decisions on timely virulence generation, to compete with other members of the community in multicellular biofilms, and to communicate and interact with other biofilm species (19). The action of QS systems is also subject to the prevailing environmental conditions (14, 20). It is sensitive to environmental factors such as iron and oxygen availability, nutritional conditions, and antibiotics (21). *P. aeruginosa* uses two QS systems, which are distinguished on the type of signal molecules they use; Acyl Homoserine lactones (AHL) and 4-quinolone signals.

*P. aeruginosa* contains two hierarchical AHL signalling systems; Las and Rhl. LasI produces the signalling molecule N-3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL), which forms complexes with the LasR protein (19, 22). The signalling molecule binds to its cognate receptor LasR only when an appropriate threshold has been reached. The 3-oxo-C12-HSL-LasR complex activates the genes responsible for the production of host entry virulence factor including genes coding for elastase, proteases, rhamnolipids and exotoxins. Additionally, the 3-oxo-C12-HSL-
LasR complex also activates the second AHL signalling system Rhl by the induction of synthesis of RhlI. RhlI produces the signalling molecule N-butanoyl homoserine lactone (C4-HSL). C4-HSL binds to its cognate receptor RhlR forming the C4-HSL-RhlR complex, which binds to and activates the genes responsible for the production of rhamnolipids, PYO and control of the Type III secretions (T3S) (23, 24). As a third AHL QS system in *P. aeruginosa*, QscR has been reported. QscR has been shown to auto activate itself and can bind to different cognate signals including 3-oxo-C12-HSL from LasI. It is, therefore, thought to be involved in cross species signalling by detecting varied signals from other species in the community (25). QscR also plays the role of downregulating the QS activation by, probably, binding to 3-oxo-C12-HSL or to lasI promoter (26). Two other potential AHL- based systems; 3-oxo-C14-HSL and 3-oxo-C10-HSL, have been reported in *Pseudomonas* but their roles and signalling molecules are yet to be discovered (27).

The PQS signalling system uses the 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-heptyl-4-quinolone (HHQ) signalling molecules. The *pqsABCDE* locus synthesizes HHQ, the precursor of PQS. The enzyme PqsH converts HHQ into PQS, which binds to its cognate receptor (PqsR) forming a PQS-PqsR complex, which auto induces *pqsA*. With the help of *PqsE*, PQS induces the genes responsible for synthesis of lectins and PYO. It also plays a secondary function of iron entrapment for later delivery to siderophores (28-30).

The two systems are intricately interconnected (Figure 1.1). LasR-3-oxo-C12-HSL complex upregulates the *pqsR*, which in turn upregulates the *pqsABCDE* operon, the genes that synthesise HHQ and PQS. PQS positively regulates the RhlI to produce more C4-HSL. Conversely, the Rhl
Figure 1.1: Intricate interconnection of the AHL and Quinolone QS systems of P. aeruginosa (modified from (19, 31)). Solid thin arrows indicate upregulation or product (in case of signalling molecule interaction). Dotted thin arrows indicate downregulation. Broad solid arrows indicate release of synthesised molecules or proteins. The two systems AHL and PQS are indicated and the virulence factors produced are shown in the boxes to the left or right of the systems.

system has been shown to negatively regulate the production of PQS, whereas 3-oxo-C12-HSL from the Las system positively regulates PQS production (reviewed in (32)).

Besides the interconnection and inter-system regulation of the QS, it is tightly regulated by additional mechanisms. This is important considering the need for timely activation and production of the virulence factors and concurrently avoiding a metabolic burden in channelling resources into non-required virulence factors. Three major regulators of activation threshold have been described; Rsal, QteE and QsIA (reviewed in (19)). These regulators ensure that QS is activated only when the threshold number of cells has been reached. Rsal binds to the promoter region of lasI preventing LasR to bind, hence, negatively regulating QS activation (33). QteE
suppresses the activation of QS by actively disrupting the LasR stability and interferes with signalling via RhlR (34). QsIA regulates the Las system by binding to LasR preventing it from binding to its target proteins (35).

1.2.1 Interplay between QS and two component signalling system

The classical QS system described above is interconnected with other cell density dependent signalling systems, making the control of virulence generation more complex and complicated. One of the interconnection that has received a lot of attention is the link to two component signalling. The global activator of antibiotic and cyanide synthesis (GAC) is the most intriguing interaction of the classical QS with a two-component signalling system. Many studies have focused on Gac because of its role in the generation of virulence in a number of bacteria (36). In *P. aeruginosa*, the Gac system coordinates the actions of two important virulence sensor kinases. Firstly, RetS sensor kinase, which mediates the transition to chronic infections by interacting with GacS, which relays a signal to the GacA regulator. GacA controls the expression of RsmZ small RNAs, which regulate the binding of RsmA to mRNAs, thereby regulating gene expression of the target genes. RsmA can also directly downregulate the production of AHLs (37). Secondy, LadS controls the genes responsible for biofilm formation in a similar way of signalling via GacS/GacA (38). The binding of RetS and LadS to GacS and subsequent activation of this signalling cascade is activated by, most likely, host and environmental signals. Many of the specific environmental factors that are relayed are yet to be comprehensively identified (Figure 1.1). However, there are indications that a number of two-component systems regulate virulence generation, via direct interaction with the QS, in response to environmental factors (39-41). However, since QS activation also depends on environmental factors, the question whether GacS is also relaying environmental signals to the central QS system remains to be answered. For a
ubiquitous bacterium like \textit{P. aeruginosa}, it is imperative that it constantly surveys the environmental conditions and for this it uses up to 64 different two-component systems (42).

A number of reports are shading more light on the possible roles of other two-component systems in surveying the environment and consequently regulating the QS depending on the prevailing conditions. The PprA/PprB sensor/regulator for controlling membrane permeability has been shown to regulate QS by influencing the influx of AHL signal molecules (41). The two component systems might interact directly with the QS to control the synthesis of the signalling molecule. For instance, the two component system BqsS/BqsR, which normally regulates biofilm dispersal, was shown to modulate the synthesis of \textit{N-C4-HSL} and \textit{PQS} (40). The ParR/ParS system that is involved in multidrug resistance was reported to also modulate \textit{rhlIR} and \textit{pqsABCDE-phaAB} (43). Another novel report is that of the two component system BfmS/BfmR modulating the \textit{Rhl} system (44). Jensen \textit{et al.} (2006) reported a possible direct link of the phosphate regulon to QS and to the prevailing environmental conditions (39).

It is therefore evident that there is an intricate interaction between the QS and two-component systems besides the global regulation of the Gac and Lad systems. This is not an unexpected phenomenon considering the vast environmental factors that \textit{P. aeruginosa} has to consider when growing in different niches and when propagating pathogenicity. It can be hypothesised that the virulence factors generation is interplay between the regulation \textit{via} the direct action of QS with reference to the quorum threshold and the regulation via the QS in response to the environmental signal as communicated and modulated \textit{via} the two-component systems. The virulence generation may also result from a direct regulation of the virulence genes via the two-component systems. Another possibility could be, the relay of the environmental conditions information to the global
two component systems and subsequently to the QS system as proposed by Wang *et al.* (2013) for ParS/ParR (43).

**1.3 Carbon source mediated synergistic virulence factors generation**

Nutritional conditions *via* the QS system control the generation of cell surface virulence factors and, consequently, biofilm formation (45). Different carbon sources seem to activate different factors in the QS network that determine the generation of biofilms. It was noted that the differences in biofilm formation were not dependent on the levels of QS signalling molecules production, since the amounts produced were similar. Hence it is highly possible that another complex regulation that works concertedly with QS comes into play. Indeed, some carbon sources can restore the wild type swarming phenotype in a QS mutant (45).

Another virulence factor that has been reported to be produced in a nutrition dependent manner is protease. Simple carbon sources, which are readily available for the organism, repress the production of protease. Complex carbon sources on the other hand do not repress protease production. Hence, the production of proteases is a response to energy limitation. It is not clear here, whether the QS system is involved (46).

Van Riji *et al.* (2004) conducted a comprehensive study on the environmental factors influencing the production of the virulence factor phenazine by *Pseudomonas chloraphis* PCL 1391 (47). This study provides deeper insight on the direct and indirect influence of different environmental factors, which gives a glimpse of the role played by environmental factors. The environmental factors seem to interact with the upstream regulation of the genes coding for virulence factors. In this study it was confirmed that the levels of phenazine-1-carboxyamide (PCN) production
correlate with the levels of the QS signals. This study reported influence of the following factors on PCN production: carbon and nitrogen source, pH, salt stress, temperature, oxygen levels, iron concentrations, phosphate, magnesium and sulphate (47).

The prevailing nutritional conditions (carbon and nitrogen sources) necessary for growth and energy generation play an important role in virulence factors production and, hence, virulence generation (47). Phenazines, for example, contain nitrogen and their production will correlate to the available source of nitrogen. Carbon sources stimulate phenazine production sources in a growth independent manner. Stimulation of phenazine production by specific carbon sources differ with different strains. Surprisingly, the stimulation of phenazine production might not necessarily be observed with the preferred carbon source. This raises the question, of which information is the available carbon source conveying to the cell or which properties of the carbon source are impacting the cell leading to the production of more phenazines.

In *P. aeruginosa*, aromatic amino acids stimulate the production of the phenazine PYO in a medium that mimics cystic fibrosis lung. This stimulation is not only due to the fact that these amino acids are building blocks in the synthesis of phenazines, because the amino acids were also shown to influence the production of PQS (48). It is, therefore, likely that the amino acids indirectly influence the production of PYO through QS.

The signalling paths of the nutritional cues (for instance, *via* two component systems) and the QS interplay for most of the carbon sources, which stimulate virulence factors production, are yet to be comprehensively determined. However, it has been shown that *P. aeruginosa* detects nutritional cues to survey and detect the presence of competitors in polymicrobial infections and,
consequently, switches on the production of antimicrobials against the competitors (49). In this report, it was shown that it can sense the peptidoglycans produced by gram positive bacteria and produce virulence factors against the competitors. It can therefore be presumed that in more complex niches containing a wide array of microorganisms, *P. aeruginosa* might sense many other molecules produced by competitors. However, in such complex niches, antimicrobials may benefit other communities, which might be capable of withstanding and utilising them.

Recently, it was shown that ethanol from *Candida albicans* stimulates the production of phenazines by *P. aeruginosa* and phenazines stimulated ethanol production (50). In another study, elevated levels of the fermentation product 2,3-butanediol were produced in CF lungs due to, perhaps, low oxygen concentrations. Metagenomic analysis showed that the 2,3-butanediol was produced by *Streptococcus spp*, and taken up by *P. aeruginosa* and it was hypothesized that this may stimulate increased phenazine production. The phenazines may then increase the pool of electron acceptors in this oxygen limited CF lungs (51). In a similar report, the fermentation product 2,3-butanediol (2,3-BD) was shown to influence the production of phenazine in a wastewater treatment BES (52). This was confirmed as a synergistic interaction between the fermenter *Enterobacter aerogenes* and *P. aeruginosa*. 2,3-BD acted as a cue that stimulated the production of more phenazines and a shift to more pyocyanin production. This resulted in increased current production at the BES anode (53). 2,3-BD influenced the production of phenazines in a QS dependent manner as was observed later in an increase of multiple virulence factors generation when *P. aeruginosa* was cultured with 2,3-BD as the carbon source (52).
Although the actual signalling path of 2,3-BD and the other metabolites that mediate phenazine production remain to be elucidated, there is a great potential in deciphering the potential of nutrition/QS-based synergistic interactions for improving efficiency of electron transfer in BES. Understanding this nutritional signalling will enable informed steering of nutrition/metabolite based synergistic and syntrophic interactions that can efficiently oxidise organic substrates in, for example, wastewater and generate electricity. The nutrient composition of wastewater is very complex and co-cultures that perform synergistic and syntrophic interactions for efficient nutrient utilisation might be very beneficial for complete oxidation of such substrates.

1.4 *P. aeruginosa* virulence factors applicable for current generation

As already mentioned, *P. aeruginosa* uses a wide array of virulence factors arsenal to propagate pathogenicity. With the sensing and regulation via the complex QS and two-component regulation systems, it generates the virulence factors efficiently, to avoid the immune system of specific hosts, generate antibiotic resistance and adapt to diverse environmental niches. Some of these virulence factors are important in the application of *P. aeruginosa* in BES

Biofilm formation is intricately connected to other virulence factors and it plays an important role in persistence and resistance to antibiotics. To colonize different environments, engage in different niche interactions and retain its metabolic versatility, *P. aeruginosa* uses the flagella and the different pili to move depending on the conditions available. It exhibits three types of motility: i) Swimming, ii) Swarming and iii) Twitching. Swimming and swarming motilities are produced by the flagella while twitching is produced by the type VI pili. Swimming is the unorganised movement on a semisolid agar. In thick fluid films the bacteria may spread both on the surface and bottom of the agar. Swarming is an organised surface movement that is organised
into strips and whirls. In swarming, the bacteria are elongated and they move along their axis; hence the movement is more organised. Twitching motility on the other hand is produced on solid media. It is less organised compared to swarming and mostly cells move singly but often small aggregates can be observed (54, 55).

Bacterial motility plays, among others, an important role in avoiding unfavourable environmental conditions and migration (56). It is, therefore, expected that nutritional conditions might be some of the environmental conditions that may influence the motility of \emph{P. aeruginosa} and a number of reports have shown this nutritional dependency of biofilm formation (45, 55). Motility influences biofilm formation; hence is an important indicator of this process. It appears therefore that there is an intricate communication between the chemical sensors concerning the nutritional environment and the QS system culminating in a decision to make a biofilm. In this process, the motility structure flagella and pili are used to explore the niches and through chemical sensing and QS signalling a decision is made to attach (57). Flagella and pili are then used in attachment to the surfaces and a biofilm is formed (58). Depending on the movement that preceded the attachment, different structures or organisation of the biofilm will be formed (45). Since the different carbon sources assessed in this study showed differences in the activity of swimming, swarming and twitching, the resulting biofilm will also be different. In BES, biofilm formation around the electrode may enhance the transfer of electrons to the electrode especially for those microorganisms that use membrane bound proteins for extracellular electron transfer. For \emph{P. aeruginosa}, formation of the biofilm around the electrodes might reduce the distance to be covered by the redox mediators. In microaerobic systems, however, \emph{P. aeruginosa} might form biofilms around the source. These possibilities of biofilm formation are important aspects for consideration in BES.
Phenazines are among the virulence factors produced, whereby PYO is the most widely studied member of the phenazines because of its significance in disease pathology. PYO has been shown to oxidise glutathione and NADH in the host cell, and once reduced it reacts with oxygen producing reactive oxygen species. This interrupts the redox balance of the host cells causing the cells to exude sputum, which serves a major carbon source for *P. aeruginosa*. The reactive oxygen species cause oxidative stress in the host cell and are thought to act as antibiotics against competing members in the niche (reviewed in (59)).

Phenazines are pigmented heterocyclic compounds. They contain a core structure composed of three 6-membered rings, with the middle ring containing nitrogen. The different phenazines arise from different substituents around the rings (Figure 1.2). Phenazines are redox active compounds, which can take up two electrons. The gained electrons can be donated to compounds with a more positive redox potential (60). The properties and reactivity of phenazines are different due to the functional group substituents, which influence the redox activity of the different phenazine species.

Phenazine biosynthesis is encoded by a conserved gene operon *phzABCDEFG* of many gram positive bacteria, which codes for the production of the intermediate phenazine-1-carboxylic acid (PCA) from chorismic acid. The differences among the phenazine species arise in the genes that convert PCA into different phenazines. The most common phenazines reported in *P. aeruginosa* cultures are PYO (blue), 1-Hydroxyphenazine (1-HP, yellow red) and phenazine-1-carboxamide (PCN, yellow) (Figure 1.2, (61)).
Figure 1.2: Overview of the biosynthesis of the common phenazines produced by *P. aeruginosa*.
Scheme (A) shows the phenazine biosynthesis genes organization into operon 1 and 2 and (B) the sequential conversion of chorismic acid into the three *P. aeruginosa* phenazines.

It is becoming more evident that phenazines play other important roles besides mediation of virulence. Interestingly, PYO also is a signalling molecule that regulates genes responsible for iron acquisition and protection from potential harm by phenazines and other QS molecules. It regulates the resistance-nodulation-cell division (RND) efflux pump MexGHI-OpmD (62). PCA plays a role in availing iron to biofilms in the late stationary phase by reducing iron III to iron II (63).

Phenazines may also play important roles in the metabolism and survival of the producers. Redox properties of phenazines may be essential for intracellular redox homeostasis control in an event
of lacking appropriate terminal electron acceptors (59). The phenazines are produced during oxygen limitation and they may be used as intermediate electron acceptors, which can shuttle electrons to extracellular terminal electron acceptors. PYO contributes to the regulation of the intracellular redox homeostasis directly by oxidising NADH. Accumulation of NADH in a phenazine mutant has been reported, which was alleviated by the addition of phenazines. In this study, it was also observed that the phenazines might be playing a role in controlling the carbon flux through the central carbon metabolism (64). This suggests that the phenazines may allow the cell to generate energy in the absence of terminal electron acceptor or in cases of oxygen limitation as it is often the case in biofilms. Since the different phenazines have different redox properties and reactivities with oxygen, they may play different roles in a P. aeruginosa biofilm and, consequently, gradients of their production exist across the biofilm structures (65). Indeed, it has been confirmed that phenazines can facilitate the survival of P. aeruginosa under anaerobic conditions (66).

It is further possible that phenazines can alter the intracellular redox balance in a non-strain specific way. In mixed microbial communities as they normally occur in the rhizosphere or in a cystic fibrosis lung, the phenazines from producers like P. aeruginosa are not only used by the producers but also by other members of the community. Thus, they might serve as common goods for redox homeostasis and other functions. This capability of phenazines is promising in applications in BES for community electron shuttling to poised electrodes.

With the increasing evidence of extracellular electron transfer in microbes for energy generation, there are many reports confirming and providing deeper insights on the role of phenazines in electron shuttling. In BES studies, it has been shown that phenazines can enable the transmission
of electrons to the electrodes, and phenazines from *P. aeruginosa* can confer electroactivity to microbial communities (16, 17, 67).

### 1.5 The BES technology

Bioelectrochemical Systems (BES) including their most important variant, the microbial fuel cell (MFC), are fast developing and promising technologies for renewable energy production and wastewater treatment among other applications (68, 69). The MFC technology aims at generating electrical current through extracellular transfer of electrons, which microorganisms liberate from organic substrates. Microorganisms oxidise organic compounds and the electrons from the intracellular electron transport chains are transferred to an external electron acceptor (i.e., an anode poised at a suitable potential) (70).

BES are technologies that utilize “biocatalysts”¹ to oxidise organic substrates for current generation. The biocatalysts may be whole cell microbes or enzymes; hence, the fuel cells may be termed as microbial or enzymatic (71). The idea of electric current generation by microorganisms was born by Potter in 1911 when he observed that bacteria would generate electromotive force when electrodes are poised in their medium (72). To generate energy, microorganisms need to discharge electrons during respiratory and fermentative metabolism. Electrons flow through the respiratory chains, drive the generation of a membrane proton gradient, and are finally accepted by terminal electron acceptors. The most common terminal electron acceptor is oxygen, a type of respiration in which microorganisms gain the highest amount of energy. Under oxygen limitation conditions, some microbes can use oxidized mineral ions and metals as external electron acceptors.

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¹ “biocatalysts” refers to microorganisms and enzymes. The term does not entirely carry the same meaning as conventional catalysts, since in the case of microorganisms, the catalyst does not only speed up the reaction, but it is part of the reaction. Microorganisms catalyse the oxidation of organic substrates and they utilize the energy gained for biomass production (Schroeder 2007).
acceptors. These are, however, less electro-positive than oxygen and they mediate generation of less energy compared to oxygen. Some microorganisms may resort to fermentation in conditions of absolute absence of an electron acceptor resulting into wasteful use of the energy source by discharging electrons into energy rich fermentation products (71). However, some of the fermentation products such as hydrogen can be used to generate electricity in an hydrogen-mediated fuel cell (73).

In MFCs, the microbes (biocatalyst) are cultured around the anode to which they can transfer their electrons. The microbes oxidise the substrates and the electrons deposited at the anode flow to the cathode. Hydrogen ions produced also flow to the cathode. At the cathode different counter reactions occur; in most MFC systems, hydrogen ions combine with oxygen to produce water (Figure 1.3-b). A higher anode potential, compared to the substrate and other available terminal electron acceptors, will make the anode a preferred electron acceptor (68, 71, 74).

In potentiostatically controlled BES, the simplest configuration is to have the working electrode (anode) and counter electrode (cathode) built into one chamber (Figure 1.3-a). A third reference electrode (RE) is installed to control the working electrode. This kind of set-up will not incur major limitations when enzymes are used as the biocatalyst. However, with microorganisms as biocatalysts, unspecific interactions may hinder the efficiency of the microorganisms in performing electrocatalysis. Alternatively, the anodic chamber can be separated from the cathodic chamber with a proton exchange membrane (PEM; Figure 1.3-b; (74)). This configuration can typically be operated as an MFC with two electrodes or potentiostatically controlled. With the reference electrode the performance of the working electrode (anode terminal) can be assessed clearly without the interference of the other parameters of the cell (reviewed in (75)).
Figure 1.3: The general working principle of MFC and a potentiostatically controlled BES. Figure (a) is a schematic representation of the single chambered BES, and (b) a scheme of the two chambered MFC separated by a proton exchange membrane.

Factors that influence the performance of MFCs may include parameters such as material used to construct the electrodes, the buffer/medium, the PEM used, and the biotic and abiotic conditions in the anodic and cathodic chambers. One of the main challenges facing the MFC performance is the efficiency of microbial electron transfer to an anode.

1.5.1 Methodology in BES

To study the system and biological changes (or responses) occurring at the working electrode specialised electrochemical tools such as the potentiostat are required. With the potentiostat a three electrode set-up can be built where the potential and current of the working electrode can be controlled. With this set-up different electrochemical tests (for instance, cyclic voltammetry (CV)) can be performed to study the electro-activity and current generation. In CV, potential scans are performed forward and in reverse direction. The resulting voltamograms provide information on the oxidation and reduction of the redox couples present in the system. CV can be used to study electron transfer mechanisms in BES and it provides useful information on the appropriate reaction potentials for an MFC (74).
Chronoamperometry (CA) is also an important method in MFCs. Here, the potential of the working electrode is controlled at a fixed value and current is measured over time. Controlling the anode potential allows the microorganisms to build a biofilm and be able to transfer electrons to it. In principal, the potential of CA should be set to the appropriate potential of the system determined by the CV measurement (74).

1.5.2 Electron transfer

The most commonly described transfer mechanisms are direct electron transfer \textit{via} direct cell contact or protein nanowires and mediated electron transfer \textit{via} secondary or primary metabolites (71, 76-80). Attempts to improve the biological efficiency of MFCs have therefore focused on understanding and improving these mechanisms.

Direct electron transfer requires direct physical contact of the cell with the electrode. Electrons flowing from the cell interior are ultimately transferred to the anode by electrochemically active outer membrane proteins (71). This type of electron transfer was reported in \textit{Geobacter} and \textit{Shewanella} (81, 82). Membrane bound \textit{c}-type cytochromes and other redox proteins aid the transfer of electrons to the anode (Figure 1.4-direct-b). It has also been reported that \textit{Geobacter} can form membrane extensions that have been termed nanowires. These extensions link the cytochromes and other redox proteins to the electrodes (Figure 1.4-direct-b; 83).
**Figure 1.4:** Schematic representation of (a) direct electron transfer and (b) mediated electron transfer (adopted from Schroeder (2007)(71)).

In mediated electron transfer, microorganisms utilize endogenous or exogenous soluble redox mediators that enable transmission of electrons to a terminal electron acceptor. Two possibilities have been described; use of secondary metabolites (Figure 1.4-mediated-a) or direct electro-catalysis of primary metabolites (Figure 1.4-mediated-b; (71). In bacteria, endogenous secondary metabolites as mediators include riboflavins in *Shewanella* (84), phenazines in *Pseudomonas* (16), and quinones in *Lactococcus* (85). These molecules undergo reversible oxidation and reduction, hence, can be used repeatedly as electron shuttles (71). Also, the addition of natural or synthetic redox compounds to enhance electron transfer in BES has demonstrated some potential
and first work shows that the heterologous expression of natural redox mediators can enable non-electroactive bacteria for electrode interactions (87). The functioning of primary metabolites as electron shuttles is based on the fact that they can be oxidised in electro-catalytic MFC to generate electricity. Metabolites such as formate and hydrogen produced during fermentation can be oxidised at catalytically active anodes (reviewed in (71)).

Phenazines produced by *P. aeruginosa* can confer electro-activity in MFCs. In mixed microbial communities, for instance, in wastewater treatment MFCs, phenazines are utilised by different species in synergistic interactions. The synergistic interaction might drive the selection of species that are cooperating (18). *P. aeruginosa* has been found to be one of the preferred producers in these communities. The synergistic interactions include co-operations that ensure efficient utilization of the organic substrates. The fermenters may breakdown complex organic substrates producing fermentation products, such as 2,3-BD, that are utilised by phenazine producers, which in turn increase the production of redox mediators (53). The fermentation product sends a signal (perhaps a nutritional cue) to *P. aeruginosa*, which most likely influences phenazine production by signalling through QS (78). This phenomenon stimulates interesting investigations into the possible role and application of nutritional based networks of interactions in BES. The phenomenon of nutritional cues and virulence generation is not new or unique to BES. There are many reports implicating nutritional conditions in influencing virulence generation by *P. aeruginosa* (48-50, 88).

### 1.6 Hypothesis and aim of the study

The reports that especially 2,3-BD stimulates the production of the phenazine virulence factors in *P. aeruginosa* motivated the investigation on the mechanisms of this stimulation (52, 53, 78). The
activation of the QS by 2,3-BD is especially important for medical and biotechnological applications; and this warrants a deeper investigation into the signalling path of 2,3-BD. 2,3-BD might be one of the nutritional cues that \textit{P. aeruginosa} uses to detected synergistic partners (or competitors). This metabolite sends a signal via QS and this mediates the elevation of production of phenazines and other necessary virulence. Here, phenazines are especially mentioned because they are beneficial to both \textit{P. aeruginosa} and the fermenter. The detection of this cue is channelled through the two component systems to the QS system or the two systems might be acting concomitantly. If this is the case, then intrinsic 2,3-BD produced by \textit{P. aeruginosa} during growth on carbon sources such as glucose may also act the same way as an autostimulant.

Alternatively, 2,3-BD might be mediating virulence generation via metabolic events that follow its catabolism or synthesis. The synthesis of 2,3-BD follows the butanoate pathway, which plays a role in regulating the amount of reducing equivalents during glucose catabolism (89). Its catabolism on the other hand involves enzymes that have been shown to be expressed under oxygen limited conditions, and thus may be linked to phenazine production, which occurs as well under oxygen limited conditions, or its catabolism under these conditions might confer energy fitness for phenazine production. Also, lack of sufficient terminal electron acceptor and, hence, intracellular accumulation of electrons, may necessitate the increased production of phenazine redox mediators.

This study purposed to decipher the possible route that 2,3-BD follows to exert influence on virulence factors production in \textit{P. aeruginosa}. It sought to answer the following questions:
i) Does 2,3-BD stimulate the production of the phenazine virulence factors, and, subsequently increased current generation, across different *P. aeruginosa* strains? (Chapter 3)

ii) How does changing the external redox environment influence growth, substrate utilisation and the production and use of the redox mediators-phenazines for current generation? (Chapter 4)

iii) Through which mechanisms and signalling pathways does 2,3-BD stimulate the elevated virulence factor production? (Chapter 5, 6 and 7)
Chapter Two

Materials and methods

2.1 *P. aeruginosa* strains

Strains PA14 (DSMZ 19882) and PAO1 (DSMZ 19880) were obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ. *Pseudomonas* sp. KRP1 was isolated from an MFC set-up at the Laboratory of Microbial Ecology and Technology (LabMET), Ghent University (deposited into the Belgian Co-ordinated Collections of Microorganisms, BCCM; strain number LMG 23160) and kindly provided to this study (18).

2.2 Media and culture conditions

Pre-cultures were grown overnight in Luria Broth (LB) medium at 37°C, then washed in 0.9% NaCl solution before BES inoculation. The electrochemical reactors were inoculated to a starting optical density OD$_{600nm}$ of 0.05. The temperature was maintained at 37°C for both cultivations in BES reactors and shake flasks. LB was prepared by dissolving 20 g of LB powder (Sigma Aldrich) per litre and was sterilised by autoclaving. For BES experiments, strains were cultured in AB medium. Procedure for preparing AB was adopted from Clark and Maløe (1967) with slight modifications. The medium contained the following constituents (per litre): component A: 2.0 g (NH$_4$)$_2$SO$_4$, 6.0 g Na$_2$HPO$_4$, 3.0 g KH$_2$PO$_4$, 3.0 g NaCl, 0.011 g Na$_2$SO$_4$, and component B: 0.2 g MgCl$_2$, 0.010 g CaCl$_2$ and 0.5 mg FeCl$_3$ $\times$ 7 H$_2$O (90). The two components were autoclaved separately before mixing them. The medium was supplemented with 30-35 mM of a single or co-feed carbon and energy source (see respective experiment).
2.2.1 *Pseudomonas Agar*

For generating mutants in chapter 6, Cetrimide Agar (Sigma Aldrich) was used to select for *Pseudomonas* from a mixture with *E. coli*. Cetrimide Agar was described by Brown and Lowbury (1965) and it has been used to isolated *P. aeruginosa* from different materials and environmental settings. It was prepared according to the manufacturer’s instructions; 46.7 g of Cetrimide were dissolved in 990 mL of water and 10 mL of glycerol was added and the agar was sterilised by autoclaving. Cetrimide Agar contains in g L-1: Gelatine peptone 20.0, MgCl₂ 1.4, K₂SO₄ 10.0, Cetrimide 0.3 and Agar 15. Cetrimide inhibits the growth of bacteria other than *Pseudomonas* by causing the extrusion of nitrogen and phosphorus (91).

2.2.2 Motility assay agar

LB agar was prepared by dissolving 20 g mL⁻¹ LB broth (Sigma Aldrich) per litre. To the LB broth, varied concentration of agar (Difco) were added; 0.3%, 0.5% and 1% for swimming, swarming and twitching, respectively. Agar was sterilised by autoclaving at 120°C for 15 minutes. After sterilisation, 30 mM of the carbon sources used in the main experiment were added. 20 mL of agar was poured into petri dishes and allowed to cool overnight. Dry plates were stored at 4°C except for 0.3% which were inoculated directly after drying since they were not solid. Cultures to be tested were inoculated using a sterile tooth pick. The plates were incubated at 37°C for 24 hours (55).

2.2.3 Synthetic cystic fibrosis medium

The synthetic cystic fibrosis medium (SCFM) was used to evaluate virulence factors production in host-like conditions or rich nutrient environment. The procedure and composition was as outlined by Palmer *et al.* (2007). All the amino acids in the proportions that they are found in the cystic fibrosis lung were added. They were suspended in the described buffer containing 1.3 mM
NaH$_2$PO$_4$, 1.25 mM Na$_2$HPO$_4$, 0.348 mM KNO$_3$, 0.122 g L$^{-1}$ NH$_4$Cl, 1.114 g L$^{-1}$ KCl, 3.03 g L$^{-1}$ NaCl and 10 mM MOPS. The concentration in mM per liter of the amino acids was as follows: 1.4 serine, 1 threonine, 1.8 alanine, 1.2 glycine, 1.7 proline, 1.1 isoleucine, 1.6 leucine, 1.1 valine, 0.8 aspartate, 1.5 glutamate 0.5 phenylalanine, 0.8 thyrosine, 0.01 tryptophan, 2.1 lysine, 0.5 histidine, 0.3 arginine, 0.7 ornithine, 0.2 cysteine and 0.6 methionine. In addition to the amino acids, 3.2 mM glucose (or 2,3-BD), 9 mM lactate and 3.6 µM FeSO$_4$ were added per liter. The medium was sterilized by filtering through a 0.2 µm filter with a vacuum (48).

2.3 Shake flask experiments

Cultivations were performed in 250 mL flasks filled with medium up to 10% of the total volume. Two conditions of aeration were used (as specified in the chapters); aerobic and microaerobic. For aerobic conditions, cultures were grown at a shaking speed of 200 revolutions per minute (rpm) and for microaerobic conditions at 80 rpm.

Pre-cultures from -80°C stocks were grown overnight in LB medium for use as inoculum. The inoculum was washed three times with equal amounts of 0.9% NaCl. The flasks were inoculated to an optical density of 0.1.

2.4 Biomass measurements

Biomass was harvested by centrifuging the culture samples at 10,000 rpm for 20 minutes. The biomass was washed once with 0.9% NaCl and transferred to pre-weighed aluminium Petri dishes. After drying overnight, the cell dry weight (CDW) was determined using a moisture analyser (RADWAG moisture analyser, Hilden, Germany).
2.5 Molecular procedures

2.5.1 PCR and Gibson assembly

The upstream and downstream regions of the genes of interest were amplified and cloned into a pEMG vector using the Gibson assembly protocol (New England labs, Ipswich, USA; the background, motivation and primers of specific knockouts are described in the specific chapters). Primers for generating templates were designed with overhang sequences for creating overlaps to join adjacent templates with help of the NEBuilder online tool for Gibson assembly (http://nebuilder.neb.com/). Gibson assembly master mix contains three enzymes; an exonuclease, a DNA polymerase and a DNA ligase. The exonuclease creates single stranded overhangs for the fragments with complementary ends to anneal. The DNA polymerase fills the gaps after annealing and the nicks that remain are sealed by the ligase.

For the cloning reaction, 50-100 ng of the vector was mixed with 2-3 fold excess inserts. Gibson assembly master mix was added and the mixture was incubated at 50°C for 15 minutes. After incubation, the products were transformed immediately into competent Escherichia coli DHαPir cells.

2.5.2 Transformation

Electrocompetent cells were prepared and stored at -80°C. E.coli DHαPir was grown overnight in LB containing no NaCl. The overnight culture was used to inoculate 50 ml of fresh LB to OD 0.05. The culture was incubated at 30°C until the OD reached 0.6-0.8. The cells were placed on ice for 5 minutes and then centrifuged. The pellet was re-suspended in 15 mL of 10% glycerol and centrifuged. The pellet was again re-suspended in 5 mL of 10% glycerol and centrifuged. The
volume of the resultant pellet was estimated and an equal volume of 10% glycerol was added. Aliquots of 50 µL were prepared and stored at -80°C.

For transformation, the electrocompetent cells were thawed on ice and transferred to a pre-chilled cuvette. The Gibson assembly cloning product was diluted 3-fold. 1 µL of the diluted product was added to the electrocompetent cells and mixed gently. Electroporation was performed using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, California, USA). The transformation mixture was incubated at 37°C with vigorous shaking at 250 rpm for 1 hour. Finally, the cells were spread on selection agar plates and incubated overnight.

2.5.3 Mutant generation using homologous recombination

Gene deletion mutants were generated via a knockout protocol published by Martinez-Garcia and de Lorenzo (2011). The protocol utilises the principal of creating breaks in the genome using I-SceI endonuclease. The pEMG vector containing I-SceI sites flanking the multiple cloning sites was used to introduce DNA segments flanking the genes of interest into the genome (Figure 2.1-a). The narrow host range origin of replication depends on the π proteins from λpir lysogens like E. coli. Therefore, in Pseudomonads the plasmid will function as a suicide plasmid, and through homologous recombination, the flanking regions of the gene of interest and the backbone are introduced into the genome. The plasmid was, therefore, used to introduce the I-SceI into the flanking regions of the gene of interest. Since the plasmid incorporates traJ and oriT, it was transformed into E. coli DH αpir and transferred to P. aeruginosa through conjugation using the helper strain E. coli DH αpir HB101. Conjugation was performed by plating the donor strain, the helper strain and the recipient (in this case P. aeruginosa) on LB Agar plates and allowing them to grow for 16 hours. Cells were collected from the plate into 0.9% NaCl and suspended.
Transformants were selected for kanamycin resistance and *E. coli* DH _αpir_ and *E. coli* DH _αpir_ HB101 were counter selected using *Pseudomonas* Agar (Chapter 2-Section 2.2.1 (92)). There are two possibilities of co-integration; to the right or left of the gene of interest (Figure 2.2).

![Diagram of pEMG and pSW-I](image)

**Figure 2.1:** a) pEMG deletion vector for introduction of flanking regions into the genome and b) PSW-1 for creating strand breaks on the I-SceI sites (adopted from (92)). For this study, a pSW-2 (92) variant containing gentamycin resistance was used.

![Diagram of co-integration events](image)

**Figure 2.2:** Introduction of the flanking regions (FR1 and FR2) of the gene of interest into the genome using the pMEG plasmid.
Finally, double strand breaks on the I-SceI site of the genome integrates were created using the vector pSW-2, which controls the synthesis of the I-SceI endonuclease. The plasmid was introduced into the P. aeruginosa co-integrates through conjugation. The strain containing this plasmid (Escherichia coli DH5 pSW-2) was grown together with the co-integrates and the helper strain on LB agar plates. Transfer of the pSW-2 plasmid to the co-integrates was confirmed by selecting for gentamycin resistance and the donor together with the helper strain was counter selected on Pseudomonas agar. Gene deletion mutants were confirmed by performing a colony PCR using primers for amplification of the two flanking regions of the gene of interest. The knockout was finally confirmed via sequencing (Figure 2.3).

2.6 BES setup and electrochemical procedures

The bioelectrochemical cell was a single chambered 500 mL glass reactor with a water jacket for temperature control (Figure 2.4). One side port with a rubber septum was used for inoculation and sampling. Top ports were used for further installations. Microaerobic conditions were achieved through two 2 µm vent filters that allowed circulation of the room atmosphere through the reactor airspace. The vent filters were flushed daily with a small air pump to remove possible condensate water from the filters. Oxygen limited conditions were necessary to generate environmental stimulus for phenazine production (53), and other virulence factors (93), but at the same time to maintain oxygen availability for directly oxygen dependent reactions such as the production of PYO (94).
Figure 2.3: Generation of double strand breaks and homologous recombination.

A three-electrode set-up was used with a comb-like solid carbon electrode (Novotec, surface area: 153 cm²) as the working electrode (anode), a carbon block (49 cm²) as counter electrode (cathode), and a saturated silver/silver chloride electrode as a reference electrode (RE, +0.187 V vs. standard hydrogen electrode at 37°C, all potentials reported vs. this reference). Electrochemical tests were performed with the Ivium-n-stat potentiostat (Ivium Technologies, Eindhoven, The Netherlands). Electric current generation was measured chronoamperometrically (CA) (in chapter 3, 0.2 V was used as the set potential and in chapter 4, varying potentials were applied). To determine the coulombic efficiency, the collected charge was related to the charge fed and consumed with each substrate with glucose: 24 e-, 2,3-BD: 22 e-, and ethanol: 12 e- per molecule.
Figure 2.4: BES set up showing the reactor and the carbon electrodes used.

Figure 2.5: Cyclic voltamograms of PCA (30 µg mL⁻¹), PYO (15 µg mL⁻¹), and a mixture of PCA and PYO (at the same concentration (left panel) and 1-HP (15 µg mL⁻¹) and PCN (15 µg mL⁻¹ (right panel) at pH 6.9.
The electrode size used for PCN and 1-HP was larger than that of PCA and PYO.
CV measurements were used to assess the electrochemical activity of the cultures every 23 hours in a potential range of -0.5 to 0.5 V at a scan rate of 2 mVs\(^{-1}\). As reference, standards of all four phenazines produced by *P. aeruginosa* were measured under similar aeration conditions in the same medium used for all experiments. The mid peak potentials of PCA and PYO individually were -0.24 V and -0.18 V, respectively. When the two compounds were mixed, the derived E\(\frac{1}{2}\) were -0.26 V and -0.18 V for PCA and PYO, respectively. The mid potentials of 1-HP and PCN were -0.253 V and -0.299 V, respectively (Figure 2.5).

### 2.7 Phenazine utilization and production analysis

To compare the specific phenazine utilisation of the three strains (chapter 3), a slightly simpler BES setup was used: AB medium with or without glucose was filled in a 100 mL standard laboratory bottle (Schott, Germany) with a septum screw cap through which two graphite rods (5 mm diameter, 5.5 cm submerged length, Novotec) and a reference electrode was installed. Each bottle was stirred at 100 rpm with a magnetic stir bar. Pre-cultured and three-times washed cells of different *P. aeruginosa* strains were added in equal cell density (OD\(_{600}\) = 1) to duplicate bottles of which one was supplied with 30 mM glucose and one without. The background current at 0.2 V applied potential was recorded for each culture for 45 min before the addition of PCA (100 µg mL\(^{-1}\) or 20 µg mL\(^{-1}\)) or PYO (20 µg mL\(^{-1}\)). Performance of the three strains in utilising the phenazines was further compared with a *P. aeruginosa* PA14 phenazine null mutant (provided by co-worker Simone Schmitz).

To gain insight into the possible differences in the genetic foundation and regulation of phenazine production by the three strains, their *phz* operons and the upstream regulatory regions were compared *in silico*. All sequence information for the genetic comparison of strains PA14 and
PAO1 was obtained from the well maintained “Pseudomonas Genome Database” at www.pseudomonas.com. The genome sequence of the BES isolate KRP1 is not published, yet. To compare the phz-gene regions of KRP1 to PA14 and PAO1, primers (Table 9.1) for the most external genes of the phz coding area were designed from the PA14 genome sequence and used to amplify the entire KRP1 phz coding area. The amplicons were sequenced by GATC, Germany. Sequence comparison for all strains was performed in Clone Manager, Scientific & Educational Software, USA.

2.8 Analytical procedures

2.8.1 Detection and quantification of phenazines

Samples of culture supernatants from chapter 3 were separated in a reverse phase column (C18 Nucleodur ec column, 250 mm long, 4 mm diameter, 5 µm particle size, Macherey & Nagel, Düren) mounted on a Shimadzu Prominence ultra-fast liquid chromatography, and detected using a photo diode array detector (Shimadzu SPD-M20A). For samples from all other chapters, the samples were separated in a waters symmetry column, Symmetry® 5µm C18 4.6x250mm (Waters, Herts, UK) mounted on a Beckman Gold HPLC (Beckman Coulter Inc, Brea, USA) also with a photodiode array detector. For C18 Nucleodur ec column, the procedure for sample analysis was as described before (61), with slight modifications. Briefly, 25 mM ammonium acetate (Fluka) as solvent A and acetonitrile (Sigma Aldrich) as solvent B were used as eluents, at a flow rate of 0.35 mL minute⁻¹ and a temperature of 20°C. A linear gradient was run for 27 minutes as follows; 5 min 20% acetonitrile, 10 min linear gradient ramp to 80% acetonitrile, 5 min 80% acetonitrile, 2 min linear gradient to 20% acetonitrile and finally 5 min 20% acetonitrile. For Symmetry®, 0.1% formic acid (pH 5) was used as solvent A and acetonitrile as solvent B at a flow rate of 0.5 mL min⁻¹. A linear gradient was run for 28 minutes as follows: 5
min 10% acetonitrile, 10 min linear gradient to 100% acetonitrile, 10 minutes 100% acetonitrile, 1 min linear gradient to 10% acetonitrile and 3 min 10% acetonitrile. Phenazines were separated and detected at their characteristic wavelengths; PYO-319 nm, PCA-366 nm, 1-HP-247 nm and PCN-247 nm. Stock solutions of phenazine standards; PCA, PCN (obtained from Princeton Biomolecular), and 1-HP (obtained from TCA Europe), were made by dissolving 1000 µg mL\(^{-1}\) of each phenazine in dimethyl sulfoxide (DMSO, Sigma Aldrich). Stock solutions of PYO (Cayman Chemical) standards were made by dissolving 2500 µg mL\(^{-1}\) in 100% ethanol.

### 2.8.2 Detection and quantification of carbon sources and metabolites

Sample supernatants were separated on an organic acid resin column (300 x 8 mm polystyrol-divinylbenzol copolymer [PS-DVB], CS-Chromatography) mounted on a Dionex Ultimate 3000 HPLC (Sunnyvale, USA). Sulphuric acid (5 mM) was used to elute the samples at a flow rate of 0.8 mL minute\(^{-1}\) and at 60°C. A refractive index (RI-101, Shodex) and UV detector (Ultimate 3000 UV/VIS detector, Dionex) at 210 nm were used to detect and quantify the different compounds depending on their properties.

### 2.8.3 AHL detection and quantification

Culture supernatants were dried under a vacuum. For the detection of C4-HSL, the dried sample was directly dissolved in 100 µL pyridine, centrifuged to remove any undissolved solids and the resulting supernatant was placed in a glass vial for measurement. For 3-oxo-C12-HSL, the dried samples were further processed using a modified protocol published by Charlton et al. (2000; (27)). The dried samples were dissolved in pentafluorobenzylxoxime (PFBO; 2 mg mL\(^{-1}\) in pyridine). They were then incubated at 37°C for 2 hours with vigorous shaking at 350 rpm. After incubation, the samples were centrifuged at high speed for 5 minutes to pellet any debris. 100 µL of the resulting supernatant were transferred into a glass vial for measurement.
Both C4-HSL and 3-oxo-C12-HSL were separated on a VF-5ms capillary column 30 m x 250 mm x 0.25 µm (coated with 1,4-bis (dimethylsiloxy)phenylene dimethyl polysiloxane) mounted on the TSQ 800 Triple quadrupole GC-MS/MS, ThermoFischer Scientific, Waltham, USA. The inlet temperature was set to 90°C and the transfer line to 300°C. An evaporation gradient from 90°C to 116°C at 10°C per second was run before transferring the sample into the column. The sample was injected using a split mode (split was set to 70). For separation, a linear temperature gradient was run from 100°C to 260°C at 10°C per minute and then from 260°C to 300°C at 20°C per minute. The MS transfer line and the ion source were set to 300°C. Data was acquired in both the scan masses and selective ion monitoring modes.
Chapter Three

Strain and substrate dependent redox mediator and electricity production by *Pseudomonas aeruginosa*


3.1 Background

A common prevalence of *P. aeruginosa* and *E. aerogenes* has been found in a mixed microbial community of a synthetic wastewater treating MFC (53). Here, further investigations have provided first insight into these interactions, which were especially pronounced under oxygen limited conditions. Redox mediators from *P. aeruginosa* were shown to mediate extracellular electron transfer in a synergistic interaction with *E. aerogenes*. The *Enterobacter* fermentation product 2,3-BD was shown to enhance and influence the spectrum of phenazine production from *P. aeruginosa*. *E. aerogenes*, in return, used the phenazines to “respire” with the electrode resulting in increased current and biomass production. There are a number of reports confirming the possible effect of certain carbon substrates in changing and enhancing the production of phenazines by *P. aeruginosa* (49, 51, 88, 95). 2,3-BD was found to not only enhance phenazine production, but also other virulence factors of *P. aeruginosa* (52). Another recent study has shown that ethanol produced by *Candida albicans* can enhance the production of phenazines by *P. aeruginosa* in polymicrobial interactions (50). These interactions represent social networks that have evolved between members occupying certain ecological niches.
In mixed microbial communities and biofilms, the redox mediators might be shared among different species and could be responsible for the syntrophic links between different species (67). Naturally, microbial communities build consortia that are characterised by intricate interactions, which often lead to a better utilisation of resources (96, 97). Some of these interactions are synergistic involving native redox mediator and non-redox mediator producers (18). Studies have shown that *Pseudomonas aeruginosa* produces phenazines that can be utilised by members of other species to transfer electrons to an external electron acceptor (16, 17). The involvement of phenazines in synergistic and syntrophic interactions among bacteria is also well documented in natural communities where, for instance, *P. aeruginosa* was found to co-exist and interact with *E. aerogenes* in marine sediments through the transfer of PYO and other metabolites (98).

Understanding this web of interactions and gaining a deeper understanding of the physiology of mediator production and usage will help us design mixed microbial cultures that effectively selfmediate electro-activity. We also need to know whether and how the interspecies interactions, redox mediator production and responses to metabolites are dependent on the strain of *P. aeruginosa*. Strains PA14 and PAO1 have been extensively used in many physiological studies. Genetically, the strains PA14 and PAO1 are appreciably different and often show discernible physiological differences. PA14 has a slightly larger genome than PAO1 (~6.54 and 6.26 MB, respectively), which contains several pathogenicity islands that are not present in PAO1 (99, 100). First pure culture evaluations of *P. aeruginosa* in BES have been performed with strain PA14 (53, 78, 101), however, this strain differs from the ones evolved in MFC microbial communities and might show a different physiological and ecological behaviour.
To elucidate the spectrum of mediator-based bioelectrochemical activity of *P. aeruginosa*, I here investigated the production of phenazines and the related current generation of three *P. aeruginosa* strains provided with different ecologically relevant carbon substrates. The well-described strains PA14 and PAO1 were examined and compared to *Pseudomonas* sp.- KRP1, which was previously isolated from a mixed microbial community of an MFC and showed 95% identity with *P. aeruginosa* in a 16S rDNA analysis (18). This evaluation forms an important foundation for future more complex co-culture investigations of mediated electron transfer in microbial fuel cells.

### 3.2 Experimental set up

The strains were cultured in AB medium supplemented with glucose, 2,3-BD, ethanol or an equimolar co-feed of glucose/2,3-BD in BES setup. Electroactivity was monitored by performing cyclic voltammetric and chronoamperometric measurements. Carbon source utilisation, final biomass generated and phenazine production were monitored and compared to electroactivity (Figure 3.1).
Figure 3.1: Experimental approach for the comparison of the substrate dependent current and redox mediator production by the *P. aeruginosa* strains in BES.

Table 3.1: Consumption rates of the three carbon substrates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate consumption rate (mM day⁻¹)ᵃ</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>2,3-BD</td>
<td>Ethanol</td>
</tr>
<tr>
<td>PA14</td>
<td>D0-D5: 3.8</td>
<td>D0-D4: 5.6</td>
<td>D0-D9: 2.2</td>
</tr>
<tr>
<td></td>
<td>D5-D11: 1.4</td>
<td>D4-D12: 1.7</td>
<td></td>
</tr>
<tr>
<td>KRP1</td>
<td>D0-D4: 4.1</td>
<td>D0-D4: 6.6</td>
<td>D1-D5: 5.8</td>
</tr>
<tr>
<td></td>
<td>D4-D10: 2.0</td>
<td>D4-D8: 2.3</td>
<td>D5-D9: 1.3</td>
</tr>
<tr>
<td>PAO1</td>
<td>D0-D10: 3.1</td>
<td>D1-D5: 3.9</td>
<td>D0-D3: 3.6</td>
</tr>
<tr>
<td></td>
<td>D5-D14: 1.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ᵃ calculated from the means of the substrate concentrations over time –Figure 3.2; D represents days of the experiment to which the rate applies

3.3 Results

3.3.1 Single substrate metabolism

The substrates glucose, 2,3-BD and ethanol were supplied at an initial concentration of 30-35 mM; by the end of the experiment all substrate was consumed (Figure 3.2). Often, an initially
high substrate consumption rate was observed up to day 4 or 5. The uptake rate strongly decreased for the later times of the experiment (to only 25-50% of initial rate, Table 3.1).

From the provided substrates, strains PA14 and KRP1 produced low levels of 2-ketogluconate (as direct oxidation product from glucose only), acetate, and acetoin as primary products, which were subsequently re-consumed (Figure 3.2-d, e and f). Production of acetoin from glucose may be a carbon-storage strategy as well as aimed at preventing the pH effects of possible acidic products (102, 103). The direct interconversion of 2,3-BD to acetoin likely caused the increased production of acetoin during growth with 2,3-BD as the carbon source (104). Strain KRP1 also produced succinate during growth with glucose as carbon source, which was also later consumed (Figure 3.2). Succinate is one product of the pyruvate fermentation pathway, which was shown to be activated for anaerobic survival of *P. aeruginosa* (105), especially in the presence of phenazines to discharge surplus reducing equivalents (106). This indicates that the cells were oxygen limited due to high metabolic activity (for example, biomass production) during days six to eight. In contrast to PA14 and KR1, hardly any substrate degradation products or intermediates were found for strain PAO1.
Figure 3.2: Carbon substrate consumption (a, b, c) and metabolite formation time profiles (d, e, f) of three *P. aeruginosa* strains in oxygen-limited bioelectrochemical experiments; with *P. aeruginosa* strain PA14 on top, *Pseudomonas* strain KRP1 in the middle, and *P. aeruginosa* strain PAO1 at the bottom.

All data are means plus standard deviations of three biological replicates, except for KRP1:2,3-BD, which only includes duplicates. The lower reported concentrations of
ethanol for PAO1 (initial concentration was also 30 mM) are due to strong evaporation during sampling and HPLC analysis.

3.3.2 Single substrate phenazine and current generation

Electric current generation by the three strains was recorded chronoamperometrically at a provided anode potential of 0.2 V vs. RE (Figure 3.3). In parallel, phenazine production during the experiments was quantified to correlate the mediators to the electrochemical activity. For strain PA14, a switch to production of PYO (22 µg mL⁻¹ vs. hardly any) and a sevenfold increased production of PCA (60 µg mL⁻¹ vs. 8.5 µg mL⁻¹) when provided with 2,3-BD compared to glucose was observed, and consequently a threefold higher current density (17 and 5 µA cm⁻² for 2,3-BD and glucose, respectively). This is comparable to the earlier report of increased maximum current density with 2,3-BD compared to glucose as a substrate (5.2 and 3.3 µA cm⁻², respectively) (53).

Compared to this, KRP1 produced its highest current with glucose as carbon source (j_max = 19 µA cm⁻²) at very high amounts of PCA in the culture (up to 150 µg mL⁻¹), while PYO (up to 17 µg mL⁻¹) was only produced very late in the experiment when the current levels already decreased (>day 11). With 2,3-BD, three times lower amounts of PCA were produced, but instead considerable amounts of PYO (20 µg mL⁻¹) at the beginning of the experiment. Under this condition, a current density of 14 µA cm⁻² was observed for strain KRP1. In comparison to glucose and 2,3-BD, both PA14 and KRP1 showed much lower electroactivity during growth with ethanol as the carbon source and this is in line with the low amounts of phenazines produced (Figure 3.3).
Figure 3.3: Current generation and phenazines production by *P. aeruginosa* PA14 (top), KRP1 (middle) and PAO1 (bottom) grown with the substrates glucose (left), 2,3-BD (vertical middle), and ethanol (right). Data originate from three biological replicates, except KRP1:2,3-BD, which was done in duplicates. Consider the different current density axis scaling for PA14:2,3-BD, KRP1:glucose, and KRP1:2,3-BD.
Table 3.2: Coulombic efficiencies of the conversion of different substrates to electric current

<table>
<thead>
<tr>
<th>P. aeruginosa strain</th>
<th>Substrate</th>
<th>Percentage</th>
<th>1:1 Glucose:2,3-BD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14</td>
<td>Glucose</td>
<td>1.8±0.3%</td>
<td>4.5±0.3%</td>
<td>1.1±0.0%</td>
</tr>
<tr>
<td></td>
<td>2,3-BD</td>
<td>4.5±0.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>1.1±0.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:1 Glucose:2,3-BD</td>
<td>2.3±0.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRP1</td>
<td>Glucose</td>
<td>8.9±0.6%</td>
<td>6.5±0.3%</td>
<td>3.4±0.7%</td>
</tr>
<tr>
<td></td>
<td>2,3-BD</td>
<td>6.5±0.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>3.4±0.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:1 Glucose:2,3-BD</td>
<td>2.6±0.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Glucose</td>
<td>0.2±0.1%</td>
<td>1.5±0.3%</td>
<td>1.1±0.1%</td>
</tr>
<tr>
<td></td>
<td>2,3-BD</td>
<td>1.5±0.3%</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>1.1±0.1%</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1:1 Glucose:2,3-BD</td>
<td>0.1±0.0%</td>
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<td></td>
</tr>
</tbody>
</table>

Table 3.3: Final bioreactor biomass as cell dry weight and biofilm tendency for different carbon sources

<table>
<thead>
<tr>
<th>P. aeruginosa strain</th>
<th>Substrate</th>
<th>Percentage</th>
<th>1:1 Glucose:2,3-BD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14</td>
<td>Glucose</td>
<td>1.29±0.06 g/L</td>
<td>0.27±0.02 g/L</td>
<td>0.48±0.09 g/L</td>
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<tr>
<td></td>
<td>2,3-BD</td>
<td>0.48±0.09 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biofilm: +++</td>
<td>Biofilm: +++</td>
<td>Biofilm: +</td>
<td>Biofilm: +</td>
</tr>
<tr>
<td>KRP1</td>
<td>Glucose</td>
<td>0.46±0.23 g/L</td>
<td>0.34±0.01 g/L</td>
<td>0.26±0.03 g/L</td>
</tr>
<tr>
<td></td>
<td>2,3-BD</td>
<td>0.34±0.01 g/L</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Biofilm: ++</td>
<td>Biofilm: ++</td>
<td>Biofilm: +</td>
<td>Biofilm: +</td>
</tr>
<tr>
<td>PAO1</td>
<td>Glucose</td>
<td>0.43±0.15 g/L</td>
<td>0.37±0.01 g/L</td>
<td>0.74 g/L</td>
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<td>2,3-BD</td>
<td>0.37±0.01 g/L</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Biofilm: +</td>
<td>Biofilm: +</td>
<td>Biofilm: +</td>
<td>Biofilm: +</td>
</tr>
</tbody>
</table>

a No cell dry weight data available; b average of two replicates; c biomass data only available for one replicate; Biofilm formation tendency is given as rough estimate with +++ strong biofilm formation on reactor liquid, ++ medium biofilm formation, + weak biofilm formation.

In contrast, PAO1 was barely electroactive with all substrates whereby the highest current was recorded with 2,3-BD ($j_{\text{max}}=4 \mu\text{A cm}^{-2}$). The low electroactivity of PAO1 concurred with low or undetectable levels of phenazines in our experiments (Figure 3.3).

The substrate based energy yield was derived as coulombic efficiency (CE) from the integral of the individual current curves over time (Table 3.2). In comparison, strains PA14 and PAO1 recorded low efficiency with glucose (1.8% and 0.2% respectively), which increased during growth with 2,3-BD (4.5% and 1.5% respectively). In contrast, KRP1 recorded higher efficiency...
with glucose (8.9%), which reduced when cultivated with 2,3-BD (6.5%). With ethanol, the
coulombic efficiencies were generally much lower compared to 2,3-BD.

Biofilm formation was visually determined and generally strain PA14 formed stronger biofilm
with most of the carbon sources compared to KRP1 and PAO1. Except for glucose, no major
differences were observed in biomass generation with all the carbon sources (Table 3.3)

3.3.3 Co-substrate consumption and bio-electrochemical utilisation

To test the functional relationship of the presence of different carbon sources, equimolar glucose
and 2,3-BD substrate were co-fed to the three strains (at 15 mM each). PA14 showed a typical
diauxic growth pattern where glucose was first consumed and depleted before 2,3-BD was taken
up (Figure 3.4-a). The measured current profile (Figure 3.4-d) also reflects the two activity
phases: the glucose-based current enters a stable plateau of ~3.25 µA cm$^{-2}$ around day 4.5. The
current starts to rise again to a maximum ~4.7 µA cm$^{-2}$ after day 7 when 2,3-BD is consumed as a
substrate. The current increase with transition to 2,3-BD coincides with a strong increase of PCA
from 0.5 µg mL$^{-1}$ to 2.5 µg mL$^{-1}$.

Similar to PA14, HPLC analysis of carbon source consumption by KRP1 depicted a diauxic
growth pattern which was, however, not easily distinguishable in the current generation (Figure
3.4-b, e). Electric current increased rapidly during the initial glucose growth phase (up to ~3.5 µA
cm$^{-2}$). During 2,3-BD consumption, the current further increased to over 10 µA cm$^{-2}$. Thereby,
strain KRP1 produced very high concentrations of PCA mainly during glucose consumption (22
µg mL$^{-1}$ by day 2; over 80% of the total PCA production in the experiment). But the highest
metabolic activity of electron discharge to the anode only took place after glucose was consumed.
Unlike PA14 and KRP1, PAO1 consumed both the carbon sources concomitantly while producing the lowest amounts of metabolic side products (<1 mM, Figure 3.4-c) and very low current densities (Figure 3.4-f). CE for the co-fed experiments was similarly low as with the single substrate experiments.

Figure 3.4: Carbon substrate uptake of cultures supplied with equimolar glucose/2,3-BD (a, b, c) and related electric current generation and phenazine concentrations (d, e, f): strain PA14 (top); strain KRP1 (middle) and strain PAO1 (bottom).
The vertical dashed lines in plots a, b, d and e) represent the time point of substrate switch from glucose to 2,3-BD. All data are means plus standard deviation of three biological replicates.

### 3.3.4 Cyclic voltammetry analysis of redox activity

Cyclic voltammetry (CV) measurements provide characteristic redox peak systems of different redox active compounds, from which specific mid-peak potentials ($E_{1/2}$) and information on the reversibility of the redox reaction can be derived.

In microbial experiments, the complex cyclic voltamograms differed from the phenazine standards (Figure 2.5) depending on the strain, carbon source, time point of the experiment and culture pH (Figure 3.5). Here, the mixed potentials can only be evaluated in combination with a chemical analysis (via HPLC) of the medium at the respective time points (Table 3.4, Table 3.5). From the daily scans, a representative CV and HPLC data for the early phase of increasing bioelectrochemical activity and during maximum detected current production is shown.

**Table 3.4: Electrochemical and chromatographic phenazine analysis for strain PA14**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>Day</th>
<th>pH</th>
<th>$E_{ox}$ (V)$^a$</th>
<th>$E_{red}$ (V)$^a$</th>
<th>$E_{1/2}$ (V)</th>
<th>Phenazines$^b$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Phenazines (µg/mL)</td>
</tr>
<tr>
<td>PA14</td>
<td>Glucose</td>
<td>3</td>
<td>6.8</td>
<td>-0.20</td>
<td>-0.43</td>
<td>-0.31</td>
<td>PYO: -5.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>6.2</td>
<td>-0.14</td>
<td>-0.26</td>
<td>-0.20</td>
<td>PCA: 5.8</td>
</tr>
<tr>
<td></td>
<td>2,3-BD</td>
<td>2</td>
<td>6.7</td>
<td>-0.17</td>
<td>-0.21</td>
<td>-0.19</td>
<td>PYO: 2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>6.7</td>
<td>-0.19</td>
<td>-0.42</td>
<td>-0.30</td>
<td>PCA: 51.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.10</td>
<td>-0.28</td>
<td>-0.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.01</td>
<td>-0.17</td>
<td>-0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>3</td>
<td>7.3</td>
<td>-0.19</td>
<td>-0.22</td>
<td>-0.20</td>
<td>PYO: 3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>6.7</td>
<td>-0.13</td>
<td>-0.21</td>
<td>-0.17</td>
<td>PCA: 10.5</td>
</tr>
</tbody>
</table>

$^a$ Oxidation and reduction peak potentials derived from the representative CVs in Figure 4; $^b$ Corresponding phenazine concentration related to the representative CV in Figure 4, not to the averaged phenazine concentrations from Figure 3; $^c$ -: No phenazines detected or a redox peak was not distinguishable.
Table 3.5: Electrochemical and chromatographic phenazine analysis for strain KRP1 and PAO1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>Day</th>
<th>pH</th>
<th>$E_{\text{ox}}$ (V)$^a$</th>
<th>$E_{\text{red}}$ (V)$^a$</th>
<th>$E_{\frac{1}{2}}$ (V)</th>
<th>Phenazines$^b$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRP1</td>
<td>Glucose</td>
<td>2</td>
<td>6.7</td>
<td>-0.18</td>
<td>-0.28</td>
<td>-0.23</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>6.2</td>
<td>-0.14</td>
<td>-0.28</td>
<td>-0.21</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2,3-BD</td>
<td>3</td>
<td>6.7</td>
<td>-0.30</td>
<td>-0.39</td>
<td>-0.34</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.22</td>
<td>-0.27</td>
<td>-0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>6.7</td>
<td>-0.12</td>
<td>-0.37</td>
<td>-0.25</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>2</td>
<td>6.7</td>
<td>-0.22</td>
<td>-0.40</td>
<td>-0.31</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>6.7</td>
<td>-0.19</td>
<td>-0.38</td>
<td>-0.28</td>
<td>-</td>
</tr>
<tr>
<td>PAO1</td>
<td>Glucose</td>
<td>2</td>
<td>6.7</td>
<td>-0.266</td>
<td>-0.385</td>
<td>-0.325</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>6.7</td>
<td>-0.19</td>
<td>-0.41</td>
<td>-0.30</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2,3-BD</td>
<td>3</td>
<td>7.1</td>
<td>-0.15</td>
<td>-0.40</td>
<td>-0.27</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.00</td>
<td>-0.26</td>
<td>-0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>3</td>
<td>6.7</td>
<td>-$^c$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>6.7</td>
<td>-0.21</td>
<td>-0.27</td>
<td>-0.24</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Oxidation and reduction peak potentials derived from the representative CVs in Figure 4; $^b$ Corresponding phenazine concentration related to the representative CV in Figure 4, not to the averaged phenazine concentrations from Figure 3; $^c$: No phenazines detected or a redox peak was not distinguishable.

A fairly broad redox peak system was found for strain PA14 supplied with glucose, which shifted from $E_{\frac{1}{2}}$=-0.31 to -0.20 from day 3 to day 12 (Figure 3.5). The HPLC analysis only indicated the presence of PCA at these time points (Table 3.4). In comparison, PA14 supplied with 2,3-BD as the carbon source showed more defined redox peak systems at day 9 ($E_{\frac{1}{2}}$=-0.30, -0.19 and -0.09 V), during which PYO and PCA were present in the culture (20 and 51 µg mL$^{-1}$, respectively). During growth with ethanol as the carbon source, a redox peak system at $\sim E_{\frac{1}{2}}$=-0.18 V was identified at day 3 and 8, corresponding to PYO as the dominating phenazine in this culture (4-times more PYO than PCA at both time points).
Figure 3.5: Cyclic voltamograms of the three *P. aeruginosa* strains (vertical variable) grown with the three carbon sources (horizontal variable). Plots show a representative blank scan (immediately after inoculation), one scan during increasing electrochemical activity and one scan during maximum electrochemical activity of one representative biological replicate for each condition.
Unlike for PA14, easily distinguishable redox peaks were observed for KRP1 supplied with glucose. Notably at day 10, KRP1 produced very high amounts of PCA (106 µg mL\(^{-1}\)), which correlated with a prominent corresponding peak system (E\(\frac{1}{2}\) = -0.21 V) (Figure 3.4). Similarly defined peak systems were observed during growth with 2,3-BD, where roughly equal amounts of PYO and PCA were present in the culture on day 3 (16 vs. 20 µg mL\(^{-1}\) for PYO and PCA, respectively). During maximum activity (day 12), PCA clearly dominated the culture and a mixed CV with a strong peak separation was measured. Low amounts of PCA were produced by KRP1 supplied with ethanol resulting in the observed redox peak system at E\(\frac{1}{2}\) = -0.28V (Figure 3.5, Table 3.4). Strain PAO1 produced very low amounts of phenazines and redox peak systems for strain PAO1 were not clearly defined.

3.3.5 Evaluating differences in usage and production of phenazines

To evaluate if the strains differ more in electrochemical behaviour because of a difference in electrochemical utilization of the phenazines or in phenazine production, two additional analyses were performed. In the first, all strains were washed thoroughly to remove endogenously produced phenazines from the cells, supplied them into medium with glucose (to generate metabolic activity) and without glucose (resting cells) and added defined concentrations of PCA or PYO similar to the ones found in the experiments. Figure 3.6 shows the relative increase in current production for different time points after the addition of the phenazines to the respective strain (current production before addition corresponds to 100%). A significant and sustained current increase was almost exclusively observed for metabolically active cells (+glucose). For all three strains, PA14, KRP1 and PAO1, the addition of 20 µg mL\(^{-1}\) PCA or PYO resulted in a similar current between 200-300% of the non-phenazine background. The addition of the observed elevated concentration of PCA (here 100 µg mL\(^{-1}\)) only resulted in an increase in
current production capability for strains PA14 and KRP1, while PAO1 was indifferent to the increased levels of PCA. For this phenazine utilization assay, a phenazine null mutant of PA14, which was kindly provided by co-worker Simone Schmitz, was also included. It was expected this strain to behave similar to the washed PA14 culture. However, while this was true for the addition of PCA at both tested concentrations, the addition of PYO yielded 35-times higher current increase with PA14Δphz compared to the PA14 wildtype.

Figure 3.6: Phenazine utilization assay with defined phenazine concentrations. The bar graphs show the relative increase in current generation (at 0.2 V) with the addition of defined concentrations of phenazines to a resuspension of washed cells of strains PA14 (a), PA14Δphz (b), KRP1 (c), and PAO1. The dashed horizontal line at 100% highlights the activity of the cells before phenazine addition. Solid coloured bars represent cultures with 30 mM glucose and checkered coloured bars represent cultures without glucose addition. Time points 1 min, 30 min, and 60 min after phenazine addition are reported.
In the second analysis, a direct genome sequence comparison between the coding and regulatory regions for phenazine synthesis of the three strains was performed. Table 3.6 summarizes the sequence similarity for all phenazine synthesis genes; and albeit at a very high sequence identity level (mostly >98%), it becomes clear that all genes also contain nucleotide mismatches, which could potentially result in different enzyme activities during phenazine synthesis. Also, all the major transcription regulatory elements for all strains and gene clusters with a focus on the upstream areas of phzA1 and phzA2 were evaluated, and no considerable differences were found (Figure 3.7). Therefore, differences in regulation are most likely driven by a different modulation of the overlaying quorum sensing regulatory network and not by any remarkable genetic differences between the strain’s phenazine gene clusters.

Table 3.6: DNA sequence identity (in %) of the phenazine genes in a pair-wise comparison of the three P. aeruginosa strains. The number in the bracket indicates the number of nucleotide miss-matches

<table>
<thead>
<tr>
<th>Gene</th>
<th>PA14-PAO1</th>
<th>PA14-KRP1</th>
<th>PAO1-KRP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>phzA1</td>
<td>98.98%, (5)</td>
<td>98.98%, (5)</td>
<td>100%</td>
</tr>
<tr>
<td>phzB1</td>
<td>98.36%, (8)</td>
<td>99.18%, (4)</td>
<td>98.77%, (6)</td>
</tr>
<tr>
<td>phzC1</td>
<td>99.26%, (9)</td>
<td>98.85%, 14</td>
<td>98.61%, 17</td>
</tr>
<tr>
<td>phzD1</td>
<td>99.36%, (4)</td>
<td>99.84%, (2)</td>
<td>98.87%, (7)</td>
</tr>
<tr>
<td>phzE1</td>
<td>99.15%, (16)</td>
<td>99.30%, (13)</td>
<td>99.42%, (11)</td>
</tr>
<tr>
<td>phzF1</td>
<td>99.52%, (4)</td>
<td>99.16%, (7)</td>
<td>99.40%, (5)</td>
</tr>
<tr>
<td>phzG1</td>
<td>99.38%, (4)</td>
<td>99.54%, (3)</td>
<td>99.84%, (1)</td>
</tr>
<tr>
<td>phzM</td>
<td>99.30%, (7)</td>
<td>99.40%, (6)</td>
<td>99.70%, (3)</td>
</tr>
<tr>
<td>phzS</td>
<td>98.10%, (23)</td>
<td>98.18%, (22)</td>
<td>99.59%, (5)</td>
</tr>
<tr>
<td>phzA2</td>
<td>98.98%, (5)</td>
<td>99.59%, (2)</td>
<td>98.98%, (5)</td>
</tr>
<tr>
<td>phzB2</td>
<td>99.18%, (4)</td>
<td>99.59%, (2)</td>
<td>99.59%, (2)</td>
</tr>
<tr>
<td>phzC2</td>
<td>99.43%, (7)</td>
<td>98.77%, (15)</td>
<td>98.85%, (14)</td>
</tr>
<tr>
<td>phzD2</td>
<td>99.20%, (5)</td>
<td>99.68%, 2</td>
<td>98.88%, (7)</td>
</tr>
<tr>
<td>phzE2</td>
<td>98.99%, (19)</td>
<td>99.15%, 16</td>
<td>99.42%, (11)</td>
</tr>
<tr>
<td>phzF2</td>
<td>99.52%, (4)</td>
<td>99.16%, 7</td>
<td>99.40%, (5)</td>
</tr>
<tr>
<td>phzG2</td>
<td>99.23%, (5)</td>
<td>99.38%, 4</td>
<td>99.85%, (1)</td>
</tr>
</tbody>
</table>

*The obtained sequences for phzC2 from KRP1 contained a 120 nucleotide gap. It is not clear yet, if this is due to difficulties in sequencing for this area or if the gene contains a 120bp deletion.*
Figure 3.7: Analysis of transcription regulatory elements of the phenazine gene clusters. All indicated transcription regulator binding sites (BS) have been identified for all three *P. aeruginosa* strains with the following consensus sequences: Lux-Box – ACGTTAGGCTTGCTGTTATGTTAGCGCCGGTGATCGACGCTGTTGTTTATGCAATCCACATCAGCGAACGGGATGAAA AACATAGCTTTCTGCGAACGACGGGCGAATGCACTATATGTAACGCCAA; LasR-BS – ACCTACCAGATCTTCTTGATGTTAGCGCCGGTGATCGACGCTGTTGTTTATGCAATCCACATCAGCGAACGGGATGAAA AACATAGCTTTCTGCGAACGACGGGCGAATGCACTATATGTAACGCCAA; QscR-BS (PhzR-BS) – ACCTGCCGGAAGGGCAGGTTGTTGCC; VqsR-BS – TCGCGCCGCGGCGCCGCG; AmrZ-BS – (i) CAAATTGCGCATGA, (ii) CAATTCGGGCAATTA.

3.4 Discussion

3.4.1 Substrate preference of *P. aeruginosa* strains

The carbon source preference of *P. aeruginosa* and the control of their utilisation in nature have been extensively explored (107, 108). Here, *P. aeruginosa* strains PA14 and PA01 and the *Pseudomonas* sp. isolate KRP1 were grown in passively aerated one-chambered BES setups (Figure 3.8) supplied with either glucose or the fermentation products 2,3-BD or ethanol. Our results suggest an enhanced uptake of the fermentation product 2,3-BD compared to glucose with a ~50% higher consumption rate for 2,3-BD with PA14 and KRP1 and a ~25% higher consumption rate for PA01 (Table 3.1). This might be linked to the dynamics of their transport rather than to carbon source prevalence. Co-feed experiments (Figure 3.4), which showed a
diauxic growth pattern (except PAO1) when both glucose and 2,3-BD are present as substrates, indicated that glucose is preferred over 2,3-BD. In a co-feed experiment, it is generally expected that the catabolite repression system will favour the uptake of the preferred substrate. It is widely accepted that *P. aeruginosa* prefers organic acids and amino acids to glucose in a sequential hierarchy (101, 108). Thereby, the catabolism of glucose, pursuing *via* Entner-Doudoroff pathway, is under the control of the catabolite repression system (107, 109). Our co-feed experiments suggest that glucose is a preferred substrate over 2,3-BD for *P. aeruginosa*. Indeed, the repression of transcription of the genes responsible for 2,3-BD catabolism by glucose has been experimentally confirmed in *B. subtilis* (the model organism for the study of 2,3-BD and acetoin catabolism) (102).

![Figure 3.8: Observed maximum current densities for *P. aeruginosa* strains PA14, KRP1 and PAO1 grown in the presence of glucose, 2,3-BD, ethanol or equimolar glucose/2,3-BD; averaged from three independent biological replicates; except KRP1:2,3-BD in duplicates. b) shows a photograph of the used bioelectrochemical setup.](image-url)
3.4.2 Strains dictate redox mediator and electric current production

Increased current generation has been reported before for PA14 in microbial co-cultures containing 2,3-BD producing microorganisms and in mono-cultures supplied with 2,3-BD as carbon source (53). Thereby, 2,3-BD was thought to interact with the QS regulatory system leading to increased production of phenazines including a switch in the phenazine spectrum towards PYO. Our experiments confirmed this behaviour for strain PA14 for the substrates glucose and 2,3-BD. In the co-feed experiments of these substrates, the observed current for PA14 resembles more the current profile of glucose; the high performance current of PA14 with 2,3-BD was not established after the transition. The produced phenazine (especially PCA) levels were much lower in this co-feed experiment than with the pure substrates. The regulatory differences in a 7-day old culture at transition to 2,3-BD consumption might have prevented the elevated production of both PYO and PCA as observed with 2,3-BD as single substrate.

What is intriguing is that the influence of this fermentation product seems to be strain dependent. Unlike PA14, KRP1 produced its highest current with glucose as carbon source, which correlated with very high amounts of PCA and hardly any PYO. In the glucose/2,3-BD co-feed experiment, KRP1 also produced high levels of PCA with over 80% of it in the initial consumption phase. But the highest metabolic activity of electron discharge to the anode only took place after glucose was consumed, hinting that indeed the high amounts of PCA in this strain are responsible for enhanced current generation. During the co-feed experiments, KRP1 produced the highest current density among the three strains. Also this corresponds to the high maximum concentration of PCA in the culture of KRP1 compared to PA14 and PAO1 (26.4, vs. 2.6 and 2.7 µg mL⁻¹, respectively, Figure 3.4-d, e, f). While the data presented here confirm the switch to more PYO
production with 2,3-BD in both PA14 and KRP1, the increased current production in KRP1 seems to be mainly mediated by PCA.

Thus, the influence of especially glucose on the phenazine spectrum appears to be different among the strains. As noted before, high amounts of succinate (~6 mM) were produced during growth of KRP1 on glucose and the re-consumption of this intermediate timely correlates with the highest observed electrochemical activity in the glucose experiments. For *P. aeruginosa* (strain PAO1) it was shown before that the highly preferred carbon substrate succinate suppresses PYO synthesis (and to a lower extend also PCA synthesis) through catabolite repression via the Crc protein (110). It should be investigated if a change in this regulatory path is responsible for the strong production of PCA and electric current in strain KRP1.

However, the concentration of phenazines cannot be linearly correlated to the amplitude of current generation. Addition of defined phenazine concentrations to washed cells (Figure 3.6) indicated a similar capacity to utilize the phenazines for PA14 and KRP1, but more investigations into the roles of the different phenazines in redox cycling are required. It should also be noted that the ratio of PCA to PYO is oxygen dependent because of an oxygen dependent monooxygenase (PhzM), which converts PCA to PYO. Thus, the instantaneous oxygen availability in our oxygen-limited experiments influenced the production of PCA vs. PYO for the evaluated substrates. Likely, the amplitude of current generation is a multivariate problem, influenced by phenazines, oxygen availability, metabolic and physiological preferences and complex regulatory networks.
In contrast, strain PAO1 barely showed any phenazine production and electroactivity in this study. The phenazine addition experiment also indicates a lower capacity to utilize PCA as redox-shuttle. These findings have important implications for the study of electrochemical activity of *P. aeruginosa*. In many laboratories, PAO1 is the model strain for *P. aeruginosa* investigations. However, the results presented here indicate that this strain might not be the best choice for *P. aeruginosa* electroactivity or BES microbial ecology research.

### 3.4.3 PCA mainly responsible for reversible redox cycling

CV measurements together with chemical analysis can be instrumental in making inferences about the redox activity and the redox species present in a BES setup. The voltamograms observed for cultures with higher concentrations of PYO (or a higher PYO:PCA ratio) depict irreversible or weakly reversible redox peaks (e.g., with strain PA14). Instead, in experiments with KRP1 where high amounts or only PCA were detected, CVs with clearly defined oxidation and reduction peaks were observed (Figure 3.5, Table 3.4). This could suggest that PYO was limited in redox cycling under the conditions of our BES. It has been postulated that, depending on the reactivity of the phenazines, PCA might be located deep in the biofilm where it is involved in iron acquisition whereas PYO occurs on the surface where it reacts with oxygen to produce reactive oxygen species as an antibiotic against competitors (65). Further, the reversible intracellular reduction of PYO might not be efficient: an earlier report demonstrated the possibility that *P. aeruginosa* lacks a NADPH:PYO oxidoreductase resulting in a limitation in redox cycling (111). Another recent report has shown that PYO can be irreversibly oxidized by the *Pseudomonas* quinolone signal PQS, a QS molecule, making it unavailable for redox cycling (112). Thus, the results here reflected in the light of these earlier reports support the hypothesis that PCA rather than PYO is more directly involved in redox cycling under oxygen limited
conditions. However, in our phenazine addition test, a similar relative current increase was observed for the three strains with the addition of 20 µg/ mL PCA or PYO, respectively, indicating a comparable capacity of the strains to utilize fresh PCA and PYO. Effects of phenazine aging and degradation are currently in investigation. In the comparison of the PA14Δphz mutant, which is not able to synthesize phenazines, to washed cells of strain PA14, a surprising 35-fold increase in current was observed with the addition of 20 µg/ mL PYO (Figure 3.6-b). Currently, this observation cannot be explained, but it points towards a suppressed PYO utilization in the strain that was already primed for phenazine production and usage. Thus, besides the high complexity of the phenazine regulation through QS cascades on the production side, also the overall utilisation of available phenazines might be strongly regulated within the cell.

3.4.4 Anodic electron discharge is a metabolic side reaction under oxygen limitation

To estimate the amount of energy recovered as charge from the substrates provided, the coulombic efficiency was calculated. In general, CE was low for all strains, showing that current production is likely a metabolic side reaction for all three strains. The main contributor to this low proportion of anodic electron discharge likely was oxygen. Under our oxygen limited conditions, the three strains showed different levels of efficiency of substrate conversion to side products (i.e. metabolic products and electric current) and biomass formation. Growth or biomass quantification during the experiments was not trivial, since varying levels of biofilm formation prevented reliable measurements of optical density or cell counts. Therefore, biomass was only assessed as total cell dry weight (planktonic plus biofilm biomass) at the end of each experiment (Table 3.3). Table 3.3 also indicates the observed tendency to form a strong biofilm on the air-liquid interface. No direct correlation between the final biomass, substrate utilisation and
metabolic side products could be derived. But likely, a strong biofilm formation on the surface of the reactor liquid will get many cells in close proximity to the headspace oxygen to discharge electrons (for example, with PA14 grown on glucose). Similarly, a low biofilm formation tendency in combination with a low general biomass formation might allow oxygen to be available for electron discharge throughout the reactor liquid (for example, all strains with ethanol). However besides this, fundamental strain differences might also largely influence CE; for example, PAO1 generally grows well under our oxygen limited conditions without the discharge of metabolic products or electrons.

Overall the observed current is a function of a wide array of factors that influence the amount of electrons and their efficiency of transmission to the electrode. If we consider similar oxygen availability, the most important contributor for efficient cell-to-electrode electron transfer is the phenazine concentration. High currents correlate with high phenazine concentrations, while PAO1 produces hardly any phenazines and consequently no anodic current. The main driver for a high concentration of phenazines thereby was the identity of the Pseudomonas strain, which determined the dominating phenazine specie and the influence or lack of influence of the carbon source on phenazine production. Both parameters might provide an ecological advantage in certain environments, also because the different phenazines might have different capacities for reversible redox cycling. In comparison to the influence of the strain identity and oxygen as a direct competitor for electrons, the amount of biomass seems to play a smaller role in influencing the phenazine-based anodic current in our experiments.
3.5 Conclusions

One approach to enhance electron transfer efficiency in MFCs is to co-culture microorganisms that can synergistically mediate current production by employing soluble redox mediators. Gaining more insight into the interactions that shape these electroactive communities will enable the definition of co-cultures that can effectively self-mediate current generation. Here, the electroactivity of three strains of the mediator producer *P. aeruginosa* was compared to deepen our understanding of this important member of such communities. Overall, the data indicate differences in the electroactivity of the three strains which, most likely, mirror their adaptability to different environments. Comparing all strains and carbon sources, it can be concluded that the BES isolate KRP1 is the most electroactive when supplied with the three carbon sources considered (Figure 3.8). Ongoing investigations of KRP1 in ecological, i.e. co-culture, context and a comparative transcriptome analysis with PA14 will shed further light into this naturally increased capability of electron discharge to an extracellular electron acceptor. Building on this work, more complex co-culture investigations of mediated electron transfer in microbial fuel cells will become possible.
Chapter Four

Electrochemical potential influences phenazine production and electron transfer and consequently electric current generation

4.1 Background

Phenazines are promising natural and synthetic redox mediators for enhancing current production in BES, and *P. aeruginosa* is one of the most active producers. *P. aeruginosa* has indeed shown a potential of being used as the phenazine producer in BES co-cultures (Chapter 1-section-3.3.2 (16, 17). Phenazine production by Pseudomonads is influenced by several biotic and abiotic factors in the ecological niches (47, 94). They play important roles in the metabolism of microorganisms in cases where the natural electron acceptor is missing or limiting. In *P. aeruginosa*, PYO has been confirmed to contribute in maintaining the cellular redox balance by oxidising NADH (113). Under anaerobic conditions, PYO redox cycling may enable *P. aeruginosa* to survive (66).

The four well known *P. aeruginosa* phenazines have fairly close redox potentials vs. a Ag/AgCl reference electrode: PCA (-240 mV), PYO (-116 mV), PCN (-140 mV) and 1-HP (-174 mV; Chapter 2-section-1.5). However, they have varying properties and reactivity to electron acceptors; suggesting that they may play different roles in, for example, biofilms. PYO reacts more readily with oxygen at neutral pH while PCA and the other phenazines are more reactive to solid electron acceptors like iron oxides and hydroxides. Hence, it was hypothesised that concentration gradients might exist in biofilms where oxygen availability-gradients prevail (65). Considering that the production of phenazines is stimulated by the prevailing environmental factors including oxygen and iron (47), it is probable that *P. aeruginosa* might produce different
gradients of these phenazines depending on need or on the electron acceptor potential or properties. In a BES set up, the important question is whether the applied electrode potential, which determines the redox environment, might influence phenazine production or the phenazine spectrum and their use in electron shuttling. Another possibility is that the applied potential might influence electron transfer strategies of the microorganism (114).

In the event of occurrence of different electron transfer mechanisms in an organism as in the case of *Shewanella oneidensis*, the available potential may influence the use of these mechanisms and subsequently a shift between them. This redox-stimulated switch in electron transfer mechanism is also associated with a change in the level of electric current production (114, 115). Hence, there is great potential in understanding how to correctly poise or regulate the electrodes in BES in order to obtain optimal metabolic state in the microorganism as well as electron transfer mechanisms.

In chapter 3, current production was evaluated at one electrode potential (0.2 V vs. Ag/AgCl_{sat}) only. Differences between the studied strains were observed in redox mediator production and usage. Further, exploration of electron transfer via phenazines and optimization possibilities was found to be necessary. The main endeavour in this chapter was to determine the influence of the applied electrode potential on redox mediator production and usage, growth and subsequent current generation in strain PA14. This study provides a roadmap into investigating the electron transfer and cellular physiological background of electric current generation in relation to the applied electrode potential. Understanding the influence of applied potential on cellular physiology and subsequent electric current generation will be instrumental in optimising cultures for efficient electron transfer.
4.2 Experimental set-up

In this chapter, the growth (dry cell weight per little at the end of the electrochemical experiment) and production of the four phenazines by *P. aeruginosa* strain PA14 was determined. Glucose was used as the sole carbon and electron donor and the electrochemical measurements were conducted in triplicates under microaerobic conditions (see Chapter 2-section-2.6). The potentials tested ranged from -0.4 V to +0.4 V (vs. Ag/AgCl\textsubscript{sat.}) in steps of 100 mV. The results obtained were grouped into cathodic and anodic potentials depending on the equivalent potential vs. standard hydrogen potential (SHE). The potentials -0.4 V, -0.3 V, and -0.24 V (with equivalent -0.213 and -0.113 and -0.053 Vs. SHE, respectively) were grouped as cathodic and -0.1 V, 0 V, 0.1 V, 0.2 V, 0.3 V and 0.4 V (with equivalent 0.087 V, 0.187 V, 0.287 V, 0.387 V, 0.487 V and 0.587 V vs. SHE, respectively) were grouped as anodic. Anodic (oxidative) behaviour was expected at potentials above -0.24 V, which is the formal potential of the most abundant phenazine (PCA) measured in chapter 3. Below this potential it was expected that there will be no electron discharge to the electrode using this phenazine.

4.3 Results

4.3.1 Phenazine production and current generation at cathodic potentials

Cathodic currents were observed at three potentials including the formal potential (-0.24 V) of the abundant phenazine PCA measured in the BES cultures (Chapter 3). At -0.4 V a reduction current was generated at the working electrode. A slight increase of negative current was observed, which reached a maximum of -16.13 μA cm\textsuperscript{-2} at day 12. Increasing the potential to -0.3 V resulted in the generation of an even stronger reduction current. The reduction current increased over time reaching its maximum at day 10 of growth. It therefore appears that the negative current generated, can be reconciled with growth (Figure 4.1).
Figure 4.1: Current generation and redox mediator production at cathodic potentials. Left row contains current generation over time and right row contains redox mediator and siderophore production. From top to bottom is a comparison of current and phenazine/siderophore generation for (a, d) -0.4 V, (b, e) -0.3 V and (c, f) -0.24 V. The current density for -0.4 V and -0.24 V is obtained from three biological replicates while that of -0.3 is from two.
To understand the influence of the applied potential on the redox mediator phenazines, the phenazines PCA, PYO, PCN and 1-HP were quantified. Further, the siderophore pyoverdine that is involved in iron acquisition was also detected and quantified. The phenazines PCN and 1-HP were below detection limit in all the cultures. The phenazines PCA and PYO were detected in varying concentrations in cultures grown at the different potentials, whereby PCA strongly dominated at all applied potentials even though the concentration at -0.4 V was very low. Very low amounts of PYO were detected for -0.4 V and -0.24 V; at-0.3 V PYO was below detection limit. Varying low concentrations of pyoverdine were produced at the different cathodic potentials (up to 11 µg mL\(^{-1}\) for -0.4 V and 15.6 µg mL\(^{-1}\) for -0.3 V respectively (Figure 4.1).

4.3.2 Phenazine production and current generation at anodic potentials

Anodic (oxidative) currents were observed at potentials more positive than the formal potential of PCA (-0.24 V). Increasing the potential from -0.24 to -0.1 V, a rapid increase in current was observed reaching its maximum after 12 days (Figure 4.2). The elevated current production at -0.1 V coincided with very high amounts of PCA compared to the cathodic potentials described above (up to 94 µg mL\(^{-1}\) for -0.1 V compared to 7, 75.2 and 38 µg mL\(^{-1}\) for -0.24 V, -0.3 V and -0.4 V, respectively).

Averagely, except for 0 V, the maximum current densities generated at anodic potentials were fairly similar. At 0.4 V maximum current density was attained early in the experiment compared to all other anodic potentials. However, the current reduced earlier (after 10 days), the time point at which most of the potentials were still reaching the peak current density. Considering the charge generated, the highest amount was generated at 0.3 V, which was closely similar to that
generated at -0.1 V. Except for 0 V, fairly similar charge amounts were generated at the other anodic potentials (Figure 4.2).

Figure 4.2: Current generation at anodic potentials
The amounts of phenazine redox mediators varied across the anodic potentials considered. Except for 0 V and elevated amounts of PCA were recorded at anodic potentials; with cultures grown at -0.1 V, 0.2 V and 0.3 V recording over 100 µg mL$^{-1}$. These are the cultures in which averagely higher current densities were generated over a longer cultivation time. This was in line with the findings in Chapter 3, where high concentrations of PCA produced by strain KRP1 were found to mediate elevated current production. Consistent patterns of pyoverdine production could not be drawn. Pyoverdine was detected at 0.1 V, 0.2 V and the highest concentration was produced at 0.4 V (Figure 4.3).
Figure 4.3: Phenazine and pyoverdine production at anodic potentials.
4.3.2.1 Cyclic voltammetric analysis

Cyclic voltammetry (CV) allows the analysis of the redox activity in the BES system. This may provide insight in the redox species employed in electron transfer by the biocatalysts. To decipher the use of redox species and the overall redox activity at different redox potentials, cyclic voltammetry measurement was run by interrupting the current measurements after every 24 hours. Redox peak systems obtained at the beginning and at the peak activity (in terms of electric current generation) of the culture were compared. To obtain a meaningful interpretation of the redox species responsible for the peak systems, the cyclic voltammetric data was further compared to the phenazine data.

Generally, two distinct redox peak systems were observed during the peak current production activity, which differed in the current intensity and the separation from each other (Figure 4.4). The mid peak potentials of the redox peak systems slightly shifted from one potential to the other. Peak system 1 (PS1) was identified for all applied electrode potentials, while peak system 2 (PS2) was only detected for applied potentials lower than 0 V (Figure 4.5, Table 4.1).

![Figure 4.4: Redox peak systems 1 and 2 (PS 1 and PS 2).](image)
Figure 4.5: Cyclic voltamograms for all cultures. They include a blank CV taken before inoculation, one taken at early stages of the culture growth and one at the peak current generation activity.
The pH of all experiments was fairly similar (Table 4.1) and thus, cannot simply explain the potential shifts. The mean formal potential of PS1 was the same as the formal potential of PCA standards (0.24 V; Chapter 2). Indeed, PCA has been detected via HPLC as dominating phenazine species in all experiments. Only at 0 V a broad, barely distinguishable PS 1 was observed, which correlates with the lowest detected concentrations of PCA. The activity of PS 1 can therefore be associated with the activity of PCA (Figure 4.5, Table 4.1). Changes in the chemical environment due to the different redox conditions in the experiments or due to adsorption processes to the electrode might have influenced the formal potential of PCA in the individual experiments.

Since PS 2 had a positive formal potential (E_{1/2}≈0.052 V), which is not close to the E_{1/2} of any P. aeruginosa phenazines, it is highly probable that this is not a peak system for a phenazine redox species (Figure 4.5). In an attempt to determine the location of PS2, a CV analysis was performed in the culture supernatants of -0.3 V and -0.1 V using clean electrodes. PS 2 disappeared in the supernatants; hence, PS 2 is a redox species that is adsorbed to the electrodes or located on the cells that form a biofilm around the electrode (Figure 4.6).
Figure 4.6: A comparison of the cultures (with cells) and supernatants of the same cultures using clean electrodes.

Table 4.1: Formal potentials ($E_{1/2}$) of the two peak systems observed at cathodic potentials, phenazine concentration and pH.

<table>
<thead>
<tr>
<th>Potential</th>
<th>$E_{1/2}$</th>
<th>Phenazines (μg mL$^{-1}$)</th>
<th>PC</th>
<th>PYO</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.4</td>
<td>-0.197</td>
<td>0.064</td>
<td>32.5</td>
<td>0.1</td>
<td>6.30</td>
</tr>
<tr>
<td>-0.3</td>
<td>-0.164</td>
<td>0.042</td>
<td>75.2</td>
<td>0</td>
<td>6.42</td>
</tr>
<tr>
<td>-0.24</td>
<td>-0.195</td>
<td>0.049</td>
<td>5.05</td>
<td>0</td>
<td>6.39</td>
</tr>
<tr>
<td>-0.1</td>
<td>-0.24</td>
<td>0.05</td>
<td>89.97</td>
<td>0.71</td>
<td>6.35</td>
</tr>
<tr>
<td>0</td>
<td>-0.21</td>
<td>$^b$</td>
<td>48.0</td>
<td>0</td>
<td>6.24</td>
</tr>
<tr>
<td>0.1</td>
<td>-0.22</td>
<td>$^b$</td>
<td>82.24</td>
<td>0.5</td>
<td>6.33</td>
</tr>
<tr>
<td>0.2</td>
<td>-0.20</td>
<td>$^b$</td>
<td>12.61</td>
<td>3.2</td>
<td>6.20</td>
</tr>
<tr>
<td>0.3</td>
<td>-0.217</td>
<td>$^b$</td>
<td>129.22</td>
<td>0.825</td>
<td>6.47</td>
</tr>
<tr>
<td>0.4</td>
<td>-0.23</td>
<td>$^b$</td>
<td>43.63</td>
<td>0.42</td>
<td></td>
</tr>
</tbody>
</table>

$^b$-not observed

4.3.3 Growth and carbon source uptake

Glucose uptake and the metabolites keto-gluconate, gluconate, acetate and acetoin were quantified during the culture period. The uptake of the carbon source during growth at most of the potentials was comparable, except for -0.4 V where glucose was not depleted. At an applied potential of 0 V, the depletion of glucose took slightly longer compared to the other applied potentials. With increasing positive electrode potentials, elevated amounts of ketogluconate were
produced, although this trend was not fully consistent (compare -0.3 V). Except for -0.4V, the levels of gluconate produced in cultures grown at all the potentials were below detection limit. Increased amounts of acetate (~8 mM) were produced at -0.24V compared to all other potentials applied (Figure 4.7).

Growth during cultivation at different applied potentials was determined as cell dry weight at the end of the experiment. Trends of biomass generation at the different applied potentials were not clear. For instance, the highest biomass amount was generated at 0.2 V followed by -0.3 V. The unclear patterns in biomass generation would be partly due to the measurement of the biomass at the end of the culture experiment, when most of the cells have attained the death phase (Table 4.2). Also, the age of cultures from which the biomass was measured varied between the experiments (10 to 30 days); hence the cultures measured were at different physiological states. Oxygen availability might have also influenced biomass generation since no active aeration was supplied. The determination of growth through biomass or OD measurement during the experiment was not possible due to biofilm formation at the air-liquid interface and around the electrodes (Figure 4.8).
Figure 4.7: Carbon source uptake at different electrode potentials and metabolite production over time. Notice that the carbon source is plotted on the left Y axis and the metabolites ketogluconate, gluconate, acetoin and acetate are plotted on the right Y axis.
Table 4.2: Growth and biofilm formation at the different potentials

<table>
<thead>
<tr>
<th>Potential (V)</th>
<th>Biomass (g L⁻¹)</th>
<th>Biofilm around electrode&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.4</td>
<td>0.50±0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>-0.3</td>
<td>0.81±0.083</td>
<td>+++</td>
</tr>
<tr>
<td>-0.24</td>
<td>0.74±0.08</td>
<td>+++</td>
</tr>
<tr>
<td>-0.1</td>
<td>0.55±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+++</td>
</tr>
<tr>
<td>0</td>
<td>0.56±0.05</td>
<td>+</td>
</tr>
<tr>
<td>0.1</td>
<td>0.57±0.01</td>
<td>++</td>
</tr>
<tr>
<td>0.2</td>
<td>0.82±0.31</td>
<td>+</td>
</tr>
<tr>
<td>0.3</td>
<td>0.72±0.14</td>
<td>+</td>
</tr>
<tr>
<td>0.4</td>
<td>0.57±0.02</td>
<td>+</td>
</tr>
</tbody>
</table>

Biomass was determined from three replicate reactors, except for two replicates.<sup>b</sup> -Biofilm intensity was visually determined around the electrode, with the following representations: + weak biofilm formation, ++ medium biofilm formation, and +++ strong biofilm formation.

---

Figure 4.8: Biofilm formation at the air-liquid interface and around the electrodes.
Representative pictures of biofilm formation: Left -0.4 V, where a dense biofilm forms at the air-liquid-interphase and right -0.24 V, where a biofilm additionally forms around the electrode.

Biofilm formation tendency was visually determined for all the cultures. At all potentials considered, a biofilm was formed at the air-liquid interphase at a considerably similar intensity. Cultures grown at negative applied potentials formed additionally a biofilm around the electrode at the liquid interphase. High biofilm intensity was observed at -0.3 V, -0.24 V, and -0.1 V (Figure 4.8; Table 4.2).
4.3.4 Overall influence of the applied electrode potential on *P. aeruginosa* physiology

The applied potential in a BES exerts an influence on the redox physiology of biocatalyst present (115-117). *P. aeruginosa* might respond by fine tuning its electron transfer mechanism. Furthermore, this might impact the central carbon metabolism and hence alter the overall respiration (64). Here, the overall influence of the applied potential on the current production, the redox species, and the growth was explored (Table 4.3).

**Table 4.3: Influence of applied potential on the physiology of *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.4</td>
</tr>
<tr>
<td>Charge (Coulombs)</td>
<td>1513.7±30.7</td>
</tr>
<tr>
<td>Coulombic efficiencya</td>
<td>-</td>
</tr>
<tr>
<td>Biomass (g L(^{-1}) CDW)</td>
<td>0.50±0.13</td>
</tr>
<tr>
<td>PCA(_{\text{max}}) (µg mL(^{-1}))</td>
<td>38.3±5.35</td>
</tr>
<tr>
<td>Pyoverdine (µg mL(^{-1}))</td>
<td>11.2±2.74</td>
</tr>
<tr>
<td>(j_{\text{max}})</td>
<td>-16.30±1.09</td>
</tr>
<tr>
<td>Time of (j_{\text{max}}) (day)</td>
<td>11</td>
</tr>
<tr>
<td>Carbon source uptake (mM day(^{-1}))</td>
<td>0.98±0.09</td>
</tr>
</tbody>
</table>

\(a\)-percentage of the coulombs supplied in the substrate that were liberated as current. For cathodic currents coulombic efficiencies were not calculated (-).
There were no major differences observed in the biomass generation in cultures grown at the different potentials (Table 4.3). As already mentioned, it can be possible that higher potentials, for instance 0.4 V (for this study), would mediate higher biomass yields according to Gibbs energy (118). This is only possible if different electron transfer mechanisms are available that can be employed at the different potentials for discharging electrons at these applied potentials. *P. aeruginosa*, however, uses mainly phenazines to reduce different external electron acceptors. Poising the electrode potential right at the formal potential of the most abundant phenazine (PCA) resulted in reduced production of this phenazines and no positive current, which is expected because current flow through this redox system is only possible if electron donor and acceptor to this system have a more negative and more positive potential, respectively, compared to its formal potential. High current densities across the potential range clearly coincided with high PCA concentrations, indicating that the dominating redox specie does not change with the applied electrode potential. For the organism, a productive usage of the PCA redox system is only possible at more positive potentials, which is also indicated by increased carbon source uptake rates at positive potentials as compared to negative potentials. It appears though that increasing the potential above 0.4 V may not be beneficial for the organism, since both the current production and the carbon source uptake was reduced at 0.4 V (Figure 4.3, Table 4.3).

Considering electroactivity, elevated amounts of charge were liberated at two potentials: -0.1 V and 0.3 V. Subsequently, high coulombic efficiencies and maximum current densities were attained at these potentials. Further, the increase of the potential from 0 V led to a faster generation of maximum current. Hence higher potentials may lead to accelerated reaction of the conversion of chemical energy to electrical energy by increasing the redox mediator recycling rate (Table 4.3).
4.4 Discussion

*P. aeruginosa* has demonstrated potential as a redox mediator producer; which can be used in improving electron transfer in BES (Chapter 3). However, to gain full potential of these redox mediators in electron shuttling and to provide optimal conditions for the producer, the BES conditions have to be optimised as well. The BES electrochemical conditions may play an important role in influencing the use and most likely the production of the phenazines. In this chapter, the electro-physiology of strain PA14 at varying negative and positive potentials was evaluated.

4.4.1 Increased electroactivity is a function of an appropriate potential

In potentiostatically-controlled BES, potentials are typically poised more positive to allow the flow of electrons from the microorganism to the anode similar to the anode situation in a microbial fuel cell, where oxygen is the typical terminal electron acceptor at the cathode. Setting the working electrode to positive potentials allows the microorganisms to discharge electrons to this electrode subject to the potential of their electron transfer proteins and redox mediators. Setting the appropriate potential with regard to the electron transfer physiology of the microorganism will enhance the flow of electrons from the microorganism to the electrode. Some microorganism may possess multiple electron discharge strategies and might use different potentials, and different potentials might influence this electron discharge differentially (114, 119).

In this study, positive, anodic currents were obtained from a potential range of -0.1 V to 0.4 V. The highest current density and charge was not obtained at the most positive potential (0.4 V) but at 0.3 V. An appreciably high current density was also obtained at -0.1 V; -0.1V is a more
positive potential compared to the substrate that was used (glucose, -0.23 vs. Ag/AgCl\textsubscript{sat}); hence, the oxidation of glucose is possible. The increased electroactivity observed at this potential may be a combination of the potential being appropriate for the oxidation of glucose as well as allowing the electron-transfer activity by phenazines. The electron transfer here was largely mediated by PS1 (corresponding to PCA). It is however not clear how PS 2 influences electroactivity at this potential. At 0.3 V, the potential is more positive allowing a higher rate of the flow of electrons, and \textit{P. aeruginosa} produces more redox mediators (PCA) that shuttle electrons. It has been observed in other electricigens that the applied potential may influence the electron transfer physiology and even the metabolism of the current producer (114, 115). Data in this chapter suggests that the applied potential may influence the levels of, especially, PCA produced. At potentials above -0.24 V more PCA is produced for redox cycling (Table 4.3).

At lower cathodic potentials, the electron transfer to the electrode seems to be impaired. Considering PCA (the most abundant phenazine) as the main electron transfer route to the electrode, it would be expected that at and below -0.24 V no positive (anodic) current will be generated. Indeed at -0.24 very low amounts of PCA were detected and the cultures were barely electroactive. A cathodic reduction current was recorded a-0.24 V -0.3 V and -0.4 V. In this case, the electrode might have reduced the PCA redox mediator, which donated these electrons to suitable electron acceptors. Whether this reduction current was biologically utilized, is an important question to be answered. There is an indication that the current increased over time and reduced during the death phase, which might coincide with the growth of the culture and phenazine (PCA) production. Besides the microbial cells, also oxygen likely served as electron acceptor for these electrons in our study. It is widely accepted that \textit{Pseudomonas} phenazines mediate electron transfer to distant oxygen in oxygen limited environments (59, 66, 113).
Overall, below -0.1 V the oxidation of glucose is impaired; depicted in the reduced uptake rates, which might point to a lack of required electron acceptor under these conditions, where the phenazine redox mediators are not available to discharge metabolic electrons to the electrode.

Poised electrodes may influence the energy gain of the microorganisms that are using it as a terminal electron acceptor. The energy gained from shuttling electrons to an electrode may be used by the microorganism to generate more biomass (reviewed in (117)). In this study, biomass generation across the potentials considered increased with increase in potential to more positive or negative. This may imply that, most likely, the applied potential influenced biomass generation.

Another important growth aspect in BES is the biofilm formation around the electrode. For BES microorganisms that depend on direct electron transfer, it is paramount that they are attached to the electrode. Even though *P. aeruginosa* is not known to depend on direct electron transfer, data obtained in this study suggest that negative potentials allow the formation of a biofilm around the electrode at the liquid interphase (to a higher degree -0.24V). Interestingly, at these potentials, an additional redox peak system was observed with a more positive formal potential than that of PCA. Since the peak disappeared in the supernatant, it is likely that PS 2 is a redox species that is located in the electrode or the biofilm that formed around the electrode (Figure 4.6). This may imply that *P. aeruginosa* employs additional redox species in transferring electrons to the electrode. Since its formal potential is more positive than the applied electrode potential, this redox species, however, cannot be involved in *P. aeruginosa* electron discharge but rather, possibly, in electron uptake. The electrons taken up from the potentiostat might be used to reduce other available electron acceptors for instance oxygen and minerals in the medium. It will be
interesting to find out which redox species is responsible for this additional redox peak system. An earlier report had observed an interchange between membrane cytochromes and soluble redox mediators (flavins) in *Shewanella* (reviewed in (119). Overall, the results show that the applied potential influenced the electron transfer physiology, which might also be linked to carbon metabolism physiology. This implies that, for every BES investigations, it is important to determine the appropriate potential, which will be beneficial in steering the physiology of the biocatalyst for efficient current generation. From the data presented here, it can be concluded that 0.3 V was the most appropriate potential under the conditions of the BES used. 0.3 V allows a rapid generation of maximum current density, more charge and overall higher coulombic efficiency is attained.

### 4.5 Conclusion

*P. aeruginosa* has demonstrated potential as a redox mediator producer for application in current generation in BES. To harness the full potential of the production of these mediators and their usage in electron shuttling, it is imperative that the important factor of the applied potential is well understood. In this chapter the analysis of the electroactivity of *P. aeruginosa* PA14 at different potentials reveals a profound influence of the applied potential on the levels and rate of current production. Higher potentials increase the rate of peak current generation. These variations in levels of current generation are a result of different concentrations of the PCA redox mediators detected at the different potentials. Further, *P. aeruginosa* activates an additional redox species at lower potentials, of which activity and specific role is yet to be identified.
Chapter Five

Quorum sensing activation and virulence factors production by *P. aeruginosa* in different nutritional backgrounds

5.1 Background

The influence of nutrition on virulence has been reported for a number of nutritional components (49, 50, 88). Some of the nutritional components that enhance virulence are thought to provide surveillance tools for the detection of competitors (49). Besides 2,3-BD, ethanol, cell wall components of some gram positive bacteria and aromatic amino acids, an extensive study of other carbon sources and environmental factors revealed the intricate interaction between nutritional conditions and the production of the virulence factor phenazine in *Pseudomonas chlororaphis* (47, 50, 52, 88). In some of these reports, the influence of the nutritional components has been linked to the increased activation of the QS system; hence, their influence is not based on growth but rather a stimulatory effect. The question arises whether the fermentation product 2,3-BD sends a signal of the presence of competitors (or synergistic partners) via QS signalling, and *P. aeruginosa* responds by producing more phenazines, or other necessary virulence factors.

However, since the 2,3-BD synthesis contributes to the regulation of NADH-NAD ratio, it is also possible that the presence of 2,3-BD in the growth medium provides additional reducing equivalents besides those gained from the other carbon sources that are consumed concomitantly. If the latter is the case then this should be also observed in conditions and nutritional backgrounds that generate high amounts of reducing equivalents.
For most of the carbon sources that have been shown to enhance virulence, little is known on how they mediate this. To get a deeper background of the influence of 2,3-BD on virulence generation via probably QS, the virulence factor production of two selected strains of \textit{P. aeruginosa} provided with different carbon sources was compared. The virulence factor production with 2,3-BD and glucose as substrates and the underlying QS activation was evaluated. Further, to get insights on nutritional cue nature of 2,3-BD in enhancement of virulence (Chapter 3), the results of 2,3-BD and glucose were compared with carbon sources of varying properties, and mimicking both environmental and host niches. This characterisation was performed in shake flasks, since the observations in chapter 3 were made in BES under the influence of the electrode.

The BES conditions might provide unique conditions in terms of electron discharge due to influence of the electrode as well as aeration. This detailed characterisation of QS and virulence generation in different nutritional backgrounds provides insight towards understanding how the nutritional cues mediate virulence generation in \textit{P. aeruginosa}. It will provide clues whether the nutritional cues, as a rule, differentially activate QS or may directly (also via other regulatory mechanisms) influence virulence generation, for instance, as in the case of high reducing equivalents necessitating increased amounts of redox mediators.

\textbf{5.2 Experimental set up}

The production of three virulence factors (phenazines, pyoverdine, and rhamnolipids) and the central QS signals was determined in mineral medium containing four different carbon sources. The mineral medium mimicked the environmental niche that is often characterised with limited nutrients. The cultures were grown under two aeration conditions: aerobic (250 mL flasks with 25
mL medium were shaken at 200 rpm) and microaerobic (250 mL flasks with 25 mL medium were shaken at 80 rpm; Figure 5.1). *P. aeruginosa* formed aggregates and a biofilm on the walls of the culture flasks; hence, growth could not be determined by measuring the optical density. Therefore, growth was determined as CDW by sacrificing triplicate cultures at every sampling point. In chapter 1, 2,3-BD and glucose were provided in cultures grown in BES to assess their redox mediator production and subsequent current production. A comparison of the redox mediator and other virulence factors production revealed that 2,3-BD influenced their production in a strain specific manner. Here, the carbon sources used in BES were evaluated in shake flasks for *P. aeruginosa* strains PA14 and KRP1. To determine the nutritional cue nature vs. QS stimulation of 2,3-BD, two more carbon sources with different properties were also included. The comparison included the preferred substrate succinate and the fatty acid octanoate (Figure 5.1). Phosphatidyl choline is thought to be one of the main carbon sources in the cystic fibrosis lung and fatty acids like octanoate form a large percentage of its composition. Further, fatty acid degradation has been associated with virulence generation (120-122).

**Figure 5.1:** Schematic representation of the experimental design for cultivation of *P. aeruginosa* strains PA14 and KRP1 in media containing different substrates.
The influence of 2,3-BD on virulence factors production was further tested in nutrient rich medium SCFM, which mimics the cystic fibrosis lung medium. Here, strain PA14, which is highly adapted to host conditions, was cultured in this medium supplemented with either 2,3-BD or glucose.

5.3 Results

5.3.1 Growth and virulence generation in minimal medium

5.3.1.1 Glucose and 2,3-BD

5.3.1.1.1 Carbon source metabolisation

Like for the BES cultures, the substrates glucose and 2,3-BD were supplied at an initial concentration of 30 mM. Glucose was rapidly taken up and the metabolites ketogluconate, acetoin and acetate were produced. Notably, strain KRP1 produced more than 5-times more ketogluconate under both aeration conditions tested. Both strain PA14 and KRP1 consumed glucose faster under aerobic conditions than microaerobic. There were no discernible differences in the rate of glucose uptake among the two strains. Unlike glucose, 2,3-BD was preceded with a lag phase before it was taken up. During metabolism of 2,3-BD, acetoin was produced as the only metabolite. This lag phase was more pronounced in PA14 (up to 8 hours) compared to KRP1 (for 4 hours). Somewhat slower uptake rates of 2,3-BD as compared to glucose were observed for both the strains under both aerobic and microaerobic conditions. Strain PA14 converted 2,3-BD into more than 5-times more acetoin compared to KRP1. The data here indicates an overall slightly faster uptake rate of glucose than for under aerobic conditions compared. However, in the
BES set-up it was observed that even though 2,3-BD is preceded by a lag phase, faster rates are observed once the strains start to consume it. This may have resulted from the differences in oxygen levels and the availability of an additional electron acceptor in BES (the electrode; Figure 5.2, Figure 5.3). Under microaerobic conditions the uptake rates of these two carbons sources were comparable.

Figure 5.2: Glucose (a, b) and 2,3-BD (c) utilization during growth under aerobic (A) and micro-aerobic (M) conditions
Above: strain PA14 (a) glucose and (b) 2,3-BD. Below: strain KRP1 (c) glucose and (d) 2,3-BD. The metabolites keto-gluconate, acetoin and acetate are plotted on the right y-axis.
For 2,3-BD, which was preceded by a lag phase, the uptake rates were calculated after the lag phase.

In an attempt to elucidate virulence generation resulting from regulatory events due to co-presence of sugars and fermentation products, an equimolar co-feed experiment of glucose/2,3-BD was performed (in analogy to Chapter 3-section 3.3.3). It was anticipated that the metabolisation of these carbon sources will follow a diauxic pattern like it was observed in the BES reactors. Indeed, both strains consumed the two carbon sources in a diauxic manner. Strain KRP1 depleted 2,3-BD earlier (24 hours and 48 hours for aerobic and microaerobic, respectively) compared to PA14 (72 hours for both aerobic and microaerobic), and the low levels of acetoin produced by KRP1 may indicate its rapid uptake during metabolisation. Overall, the metabolisation of the carbon sources in the co-feed, particularly 2,3-BD, seems to vary in rate and product spectrum between PA14 and KRP1 (Figure 5.4).
Figure 5.4: Carbon source utilisation in a co-feed of equimolar glucose/2,3 BD mixture, and the metabolites produced under aerobic (A) and microaerobic (M) conditions for strains: (a, b) PA14 and (b, c) KRP1.

5.3.1.1.2. Biomass generation with glucose and 2,3-BD as the carbon sources

Growth was measured as cell dry weight per litre of medium. Biomass was determined in three biological replicate flasks that were sacrificed at respective time-points. Since the biomass time profile was appreciably different for the substrates, growth rates were determined only during the exponential phase.

Cultures provided with glucose as the carbon source grew rapidly and strain KRP1 generated the highest peak biomass under both aeration conditions. Growth with 2,3-BD as the carbon source
showed a short (8 hours) lag phase at the beginning. Unlike glucose, with 2,3-BD as the carbon source strain PA14 generated the highest peak biomass under aerobic conditions (Figure 5.5b). Considering the growth rates, strain KRP1 showed an exceptionally high (more than 5 times higher than the second highest) growth rate when provided with glucose under aerobic conditions. Averagely, lower growth rates were observed in cultures provided with 2,3-BD (Figure 5.5).

Figure 5.5: Growth curves of *P. aeruginosa* strains PA14 and KRP1 in medium containing glucose and 2,3-BD.
Graphs show biomass generation as cell dry weight with; (a) glucose, (b) 2,3-BD, (c) equimolar mix of glucose/2,3-BD, and (d) calculated growth rates. The letter A and M denote aerobic and microaerobic conditions, respectively.
In medium containing equimolar mixture of glucose/2,3-BD, KRP1 generated relatively more biomass compared to PA14. Considering growth rates, co-fed cultures showed higher growth rates compared to cultures provided with 2,3-BD only. Generally, the highest growth rates were observed in cultures provided with glucose only followed by those provided with the equimolar mix and lastly those provided with 2,3-BD only (Figure 5.5).

5.3.1.2 Succinate and octanoate

5.3.1.2.1. Carbon source consumption

To get additional hints of the possible action of nutritional cues on virulence generation, two more carbon sources: succinate and octanoate were assessed. Succinate has been reported as one of the preferred substrates by *P. aeruginosa* (101, 122). As already mentioned, fatty acids form one of the most common sources of nutrition under host conditions (120, 121). To represent fatty acids, octanoate was included in the comparison. Succinate was rapidly consumed by both PA14 and KRP1. Fairly similar uptake rates were observed under aerobic and micro-aerobic. With succinate as a substrate, no secreted metabolic by-products were detected. Octanoate was also consumed at comparable rates by both PA14 and KRP1. The rate of octanoate consumption was, averagely, two times lower than that of succinate (Figure 5.6, Figure 5.7).
Figure 5.6: Succinate (left) and octanoate (right) consumption over time by strain. The letter A and M denote aerobic and micro-aerobic conditions, respectively. The pH of succinate cultures changed drastically and it is plotted on the succinate graph on the right y-axis.

Figure 5.7: Calculated carbon source uptake rates of octanoate and succinate. The growth rates were determined during the exponential phase. The letter A and M denote aerobic and micro-aerobic conditions, respectively.
Since the carbon sources provided have different properties and may be differentially metabolised by the strains, it was expected that their uptake and metabolite production profile would be different. Overall, the data here indicated that succinate was the most rapidly consumed carbon source of all the carbon sources considered followed by glucose. Regarding the rate of uptake, 2,3-BD was the third and lastly octanoate (Figure 5.2, Figure 5.3, Figure 5.6, Figure 5.7).

5.3.1.2.2. Growth in medium provided with succinate and octanoate

In medium provided with succinate, both strains grew rapidly at the beginning. However, the amount of biomass generated was relatively lower compared to all the other carbon sources. Cultures provided with octanoate generated relatively higher biomass compared to succinate and 2,3-BD. The amounts of biomass generated with octanoate were comparable to those of glucose. It was also observed that with octanoate as the carbon source, strain KRP1 produced relatively higher biomass amounts compared to PA14 (Figure 5.5, Figure 5.8). Considering the growth rates, cultures provided with octanoate grew faster compared to those provided with glucose (Figure 5.9).

![Figure 5.8: Growth curves of P. aeruginosa strains PA14 and KRP1 in medium containing (left) succinate and (right) octanoate. Letters A and M denote the two conditions considered; aerobic and micro-aerobic conditions, respectively.](image)

93
5.3.1.3 Nutritional background influences virulence factor generation

5.3.1.3.1. Glucose and 2,3-BD

To determine the role of the nutritional background and, specifically the fermentation products in virulence factors production, phenazines, siderophore-pyoverdine and rhamnolipids were quantified in all conditions of growth. To also have a view of the physiological tendency to form biofilm, motility assays were performed.

Production of phenazines was quantified in triplicates over time and only PCA and PYO were above the detection limit (for time resolved profiles see Appendix 1-section 9.1). Considering
that the phenazine production time profiles and growth were different across the carbon sources, the strains and the two aeration conditions, the maximum phenazine concentrations per cell dry weight were calculated for evaluation (Table 5.1).

Under aerobic conditions, PCA production by strain PA14 in medium containing glucose and 2,3-BD was comparable. Elevated amounts of PCA in medium supplied with equimolar mix of glucose and 2,3-BD were observed in strain PA14. It was anticipated that the cultures will produce elevated amounts of PCA (or phenazines in general), as a strategy to deal with oxygen limitation. This was indeed the case for microaerobic PA14 cultures provided with glucose and 2,3-BD and their co-feed. For the latter, the PCA production under microaerobic conditions even doubled. The pattern of PCA production among the strains was slightly different. Under aerobic conditions KRP1 produced lower amounts of PCA in medium containing glucose compared to 2,3-BD. Like for PA14, PCA production for glucose and 2,3-BD cultures under microaerobic conditions was similar. In contrast, lower amounts of PCA in medium containing 2,3-BD compared to glucose for this strain was observed in (microaerobic) BES reactors (Chapter 3-section 3.3.2). Elevated amounts of PCA production in co-fed cultures (as was the case with PA14) was, however, not observed for KRP1 (Table 5.1). Overall, the amounts of PCA produced here, especially for KRP1, were much lower compared to those produced in the BES reactors containing glucose or 2,3-BD. This might have resulted from the differences in aeration and growth (mostly floating biofilm for reactors vs. planktonic growth followed with suspended aggregates in shake flasks; Chapter 3-section 3.3.2).
Table 5.1: Maximum phenazines production by strain PA14 and strain KRP1 normalized per biomass

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Aerobic PCA(^a)</th>
<th>Microaerobic PCA(^a)</th>
<th>Aerobic PYO(^a)</th>
<th>Microaerobic PYO(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>29.4±3.8</td>
<td>41.5±5.4</td>
<td>5.6±0.03</td>
<td>6.7±1.5</td>
</tr>
<tr>
<td>2,3 BD</td>
<td>25.2±6.2</td>
<td>39.2±0.4</td>
<td>6.9±1.44</td>
<td>11.6±0.96</td>
</tr>
<tr>
<td>Co-feed</td>
<td>47±6.78</td>
<td>81.9±0.35</td>
<td>8.3±1.1</td>
<td>2.2±0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>6.6±2.3</td>
<td>23.7±3.2</td>
<td>1.1±0.63</td>
<td>2.6±0.49</td>
</tr>
<tr>
<td>2,3 BD</td>
<td>33.9±0.22</td>
<td>28.9±1.62</td>
<td>2.6</td>
<td>6.9±0.54</td>
</tr>
<tr>
<td>Co-feed</td>
<td>20.5</td>
<td>11.4±1.90</td>
<td>6.3±0.19</td>
<td>5.1±1.1</td>
</tr>
</tbody>
</table>

\(^a\)concentration expressed in \(\mu\)g mL\(^{-1}\) CDW\(^{-1}\)

PYO concentrations were generally 0.5 to 1 magnitude lower than PCA concentrations, which is in accordance with the BES reactor experiments (Chapter 3). However, the specific electrochemical (and likely general redox activity) of PYO is much stronger than for PCA, so that these lower concentrations should not be underrated. Here, strain PA14 produced slightly more PYO in medium containing 2,3-BD and even more in the co-fed culture under aerobic conditions. However, under microaerobic conditions the lowest PYO concentrations were observed in the co-fed cultures. Similar to PA14, strain KRP1 produced more PYO in medium containing 2,3-BD compared to glucose and even more in the aerobic co-fed culture. Like for PCA, increased PYO production was anticipated and detected under more oxygen-stringent microaerobic conditions (Table 5.1).
Figure 5.10: Pyoverdine production by strains PA14 and KRP1 in medium containing (a) glucose, (b) 2,3-BD and (c) equimolar mix of glucose/2,3-BD.

*P. aeruginosa* produces the siderophores pyochelin and pyoverdine for acquisition of iron in iron limited environments. Only pyoverdine was quantified in this study, to provide a picture of siderophore production activity under the conditions considered. Under the two aeration conditions in general, strain PA14 produced elevated amounts of pyoverdine under aerobic conditions. Even as the pattern of pyoverdine production by strain KRP1 was not fully clear under the two aeration conditions, there seems a tendency to produce more pyoverdine under microaerobic conditions. Generally cultures provided with glucose as the carbon source produced relatively more pyoverdine compared to 2,3-BD (Figure 5.10).
The production of the surfactant rhamnolipid plays an important role in virulence generation by \textit{P. aeruginosa} (123). Since its production is also under the control of the QS system, its detection and quantification can provide insight into the web of interactions that shape the virulence physiology of \textit{P. aeruginosa}. In medium containing 2,3-BD, strain KRP1 produced more rhamnolipids compared to PA14. The pattern of rhamnolipid production under aerobic and microaerobic conditions was not clearly discernible. In medium provided with glucose the highest concentration was produced by KRP1 under microaerobic conditions (236 mg L\(^{-1}\)), whereas in 2,3-BD medium fairly similar concentration under both conditions were produced by KRP1 (273 and 201 mg L\(^{-1}\) for aerobic and microaerobic, respectively; Figure 5.11).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.11}
\caption{Rhamnolipid production in minimal medium provided with (a) glucose, (b) 2,3-BD and (c) glucose/2,3-BD co-feed.}
\end{figure}
Data is presented for strains PA14 and KRP1 under aerobic (A) and microaerobic (M) conditions. The reported rhamnolipid concentration is a sum of the following mono- and di-rhamnolipids: C8-C10, C10-C10, C10-C12, Rha-C10-C8, Rha-C10-C10, Rha-C12-C10, Rha-Rha-C10-C8, Rha-Rha-C10-C10, Rha-Rha-C12-C10, Rha-C10-C10 and Rha-Rha-C10-C10.

5.3.1.3.2. Succinate and octanoate

In medium provided with succinate, strain PA14 produced lower PCA amounts compared to octanoate as well as glucose and 2,3-BD considered above, under both aerobic and microaerobic conditions. In medium containing octanoate, strain PA14 produced higher amounts of PCA compared to succinate and also compared to glucose and 2,3-BD. Comparing the strains, the PCA production pattern was slightly different. For KRP1, lower concentrations were detected in medium containing succinate and octanoate compared to PA14. With succinate, strain KRP1 produced PCA amounts that were similar with those produced with glucose. Differences were also observed in PCA production under the two aeration conditions for these additional substrates; strain PA14 showed lower PCA production under microaerobic conditions whereas relatively more PCA was detected for strain KRP1 (Table 5.2).

Table 5.2: Maximum phenazines production by strains PA14 and strain KRP1 normalized per biomass for the additional substrates succinate and octanoate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Aerobic</th>
<th>Microaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PYO&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>9.7±0.97</td>
<td>21.4±1.6</td>
</tr>
<tr>
<td>Octanoate</td>
<td>33.2±7.6</td>
<td>34±2.7</td>
</tr>
<tr>
<td>KRP1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>6.4±0.35</td>
<td>6.01.27</td>
</tr>
<tr>
<td>Octanoate</td>
<td>13.8±3.3</td>
<td>7.7±1.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>concentration expressed in μg mL<sup>-1</sup> CDW<sup>-1</sup>
Interestingly, extremely high concentrations of PYO were produced by PA14 cultures provided with succinate and octanoate under aerobic conditions but 3-5-times lower under microaerobic condition. The concentrations produced here were higher compared to glucose and 2,3-BD. With strain KRP1, however, no elevated production of PYO with these substrates was observed (Table 5.2).

Averagely, higher amounts of pyoverdine were produced in cultures provided with octanoate compared to all other carbon sources. Cultures grown with succinate produced lower amounts of pyoverdine compared to the rest of carbon sources. Strain PA14 seems to produce relatively higher amounts of pyoverdine compared to KRP1 in medium containing octanoate (Figure 5.12).

Rhamnolipid evaluation for the additional carbon sources showed that both strain PA14 and KRP1 produced elevated amounts of rhamnolipid with octanoate compared to succinate. The highest concentration of rhamnolipids among all the carbon sources was also produced with octanoate. There were no clear patterns of rhamnolipid production with succinate and octanoate with regard to strain and aeration conditions (Figure 5.13).
Figure 5.12: Pyoverdine production by strains PA14 and KRP1 in medium containing (Left) succinate and (Right) octanoate.
The letter A denotes aerobic and M-microaerobic.

Figure 5.13: Rhamnolipid production in minimal medium provided with (a) succinate, (b) octanoate.
Data is presented for aerobic (A) and microaerobic (M) conditions. The reported rhamnolipid concentration is a sum of the following mono- and di-rhamnolipids: C8-C10, C10-C10, C10-C12, Rha-C10-C8, Rha-C10-C10, Rha-C12-C10, Rha-Rha-C10-C8, Rha-Rha-C10-C10, Rha-Rha-C12-C10, Rha-C10-C10 and Rha-Rha-C10-C10.

The data presented here indicate an overall substrate dependent production of phenazines. The production of phenazines in these nutritional environments seems to slightly differ between the two strains. Differences in phenazine production under the two aeration conditions seem to also
depend on the substrate. Also, pyoverdine production seems to be influenced by the available substrate.

5.3.1.4 Overall influence of the carbon source on virulence factors production in AB medium

To get an overall overview of the influence of the carbon sources provided on the virulence generation, the amounts of each parameter were calculated as a percentage of the highest amount measured for the specific parameter and compared (Figure 5.1). Averagely, high biomass amounts were generated with glucose for both strain PA14 and KRP1. However, the highest peak biomass amount was generated by KRP1 during growth with octanoate as the carbon source. Octanoate not only mediated the generation of the highest biomass amount but also the peak PYO and pyoverdine production, which was averagely more than 3-times higher than for all other experiments. There is, hence, every indication that octanoate influences the production of virulence factors, even though not in a uniform pattern across the two strains.
Figure 5.14: Percentage of biomass and virulence factors production in comparison to the highest measured of the specific parameters. The parameters are grouped into biomass (B), phenazine-1-carboxylic acid (pca), pyocyanin (pyo), pyoverdine (pyv), and rhamnolipids (rh).
Overall, the data indicates that the substrate provided influences the virulence factors production. 2,3-BD, which stimulated increased phenazines in chapter 3, also stimulated the production of PYO compared to glucose. Besides 2,3-BD, octanoate stimulates production of PYO under aerobic conditions. The influence of the substrates on virulence factors production is subject to strain and aeration conditions. Hence, the inconsistencies in the levels of virulence factors production under the two aeration conditions might have resulted from variations in oxygen concentration.

5.3.1.4.1. Influence of substrate on Motility

The flagella and type VI pili are responsible for motility as well as biofilm formation, where they are used for attachment to surfaces (58). Motility may also contribute to fitness of the microorganism by aiding it to avoid unfavourable conditions (124). Hence motility assay may provide insights into the background sensing and signalling. Cultures grown in medium containing the different carbon sources were inoculated onto agar plates containing 0.3%, 0.5% and 1% agar for swimming, swarming and twitching motility, respectively (Chapter 2-section 2.2.2). Tables 5.3 and 5.4 show the distance covered by the two strains from the point of inoculation on the different motility plates (for pictures see Appendix 2).
Table 5.3: Distance covered from the point of inoculation by strain PA14 during swimming, swarming and twitching.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Motility assay</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Swimming</td>
<td>Swarming</td>
<td>Twitching</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.9±0.5</td>
<td>0.8±0.2</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>2,3-BD</td>
<td>2.7±0.1</td>
<td>0.4±0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose/2,3-BD</td>
<td>1.0</td>
<td>4.1</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.6±0.1</td>
<td>7.7</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>Octanoate</td>
<td>0.4±0.1</td>
<td>0.5</td>
<td>0.7±0.1</td>
</tr>
</tbody>
</table>

All distances from the point of inoculation are in centimeters

Table 5.4: Distance covered from the point of inoculation by strain KRP1 during swimming, swarming and twitching.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Motility assay</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Swimming</td>
<td>Swarming</td>
<td>Twitching</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.7±0.3</td>
<td>0.6±0.1</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>2,3-BD</td>
<td>1.7±0.2</td>
<td>0.5</td>
<td>1±0.1</td>
</tr>
<tr>
<td>Glucose/2,3-BD</td>
<td>0.6</td>
<td>0.6±0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.7</td>
<td>1.2±0.2</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Octanoate</td>
<td>0.5±0.1</td>
<td>1.5</td>
<td>0.6±0.2</td>
</tr>
</tbody>
</table>

All distances from the point of inoculation are in centimeters

The swimming distances decreased in the following order: glucose > 2,3-BD > glucose/2,3-BD > succinate > octanoate. For KRP1, a similar trend like that of PA14 was observed except for slight elevation for succinate. The swarming distances for KRP1 with glucose, 2,3-BD, and the mix of glucose/2,3-BD were 1.5-2-times shorter than those of PA14. For swarming, PA14 swarmed two times further with glucose compared to 2,3-BD. Both PA14 and KRP1 swarmed the furthest with succinate as the carbon source. Considering twitching motility, the twitching distance for the different carbon sources were close. For PA14, the furthest distances were produced with glucose as the carbon source. KRP1 on the other hand twitched the furthest with 2,3-BD (Table 5.3, Table 5.4).
The motility data presented here imply a carbon source dependent motility. This may have implications during biofilm formation. Both strains seem to be most active in swimming when provided with glucose. Also, the highest swarm activity was observed with succinate for PA14. Mixing glucose and 2,3-BD in a co-feed leads to an elevated swarm activity for PA14, which was not observed with mono-feeds of these carbon sources. The influence of the carbon source on motility is further shaped by the differences in the strains (Table 5.3, Table 5.4).

5.3.1.4.2. Influence of the substrates on the production of QS signalling molecules

The signalling molecules of the HSL system were quantified to provide an overview of the QS activation. This would provide insights into the influence of the carbon sources on this central signalling system that activates virulence factors genes. C-4-HSL was detected after 24 hours of growth in most of the cultures and data for 24, 48 and 72 hours of growth is presented. Since the levels of HSL molecules may largely depend on cell density, the concentrations were normalised per biomass.

For PA14, the highest C-4-HSL concentration was produced by cultures grown in medium containing 2,3-BD under aerobic conditions. This amount reduced over time. Under the same conditions, cultures provided with glucose produced comparable amounts after 48 hours of growth. The co-fed cultures produced C-4-HSL concentrations similar to those of glucose. Most likely, this is an effect of diauxic growth where glucose was taken up first; hence, its influence supersedes that of 2,3-BD that is take up later. Notably, cultures provided with octanoate produced lower amounts of this signalling molecule. Based on the extremely high levels of PYO produced by octanoate cultures, it would be expected that higher amounts of the signalling
molecule would also be secreted. Further, with succinate the levels of C-4-HSL were below
detection limit. For succinate, it is not clear whether the high pH influenced the concentration of
lactones. The data presented here, therefore, indicates that the substrate influences the HSL
signalling molecule secretion. The levels of the signalling molecule do not entirely correspond to
increased virulence factors production (considering phenazines and pyoverdine; Figure 5.15).
Figure 5.15: C-4-HSL production by cultures provided with different carbon sources. The data is grouped into the different carbon sources with the following abbreviations: Gluc-glucose, Gluc/BD-equimolar mix of glucose+2,3-BD, Succ-succinate and Oct-octanoate. The concentrations of these signaling molecules were below detection limit during 0-16 hours of growth.
Figure 5.16: 3-oxododecanoylhomoserine lactone production by cultures provided with different carbon sources. 3-oxo-C12 was detected after 8 hours and was detectable up to 48 hours of growth.
In comparison to strain PA14, KRP1 produced extremely low amounts of C-4-HSL. There were no major differences in the C-4-HSL production among the carbon sources considered for KRP1 (Figure 5.15).

For 3-oxo-C12-HSL, low concentrations were produced by the two strains. Overall, for PA14, higher concentrations were recorded in cultures grown with glucose as the carbon over the three time points considered. However, the highest signal concentration was observed with the equimolar mixture of glucose/2,3-BD followed by 2,3-BD (at 8 hours). KRP1 produced averagely lower signal concentrations compared to PA14 with the highest concentration being produced with glucose as the carbon source. Similar to C4-HSL, it appears that the signal concentration does not coincide with increased production of the virulence factors especially phenazines.

5.4 Growth and virulence generation in nutrient-rich conditions
The results presented above depict the physiology of *P. aeruginosa* regarding carbon source utilization and virulence factors production under nutrient limited conditions, since the minimal medium may mimic nutrient-limited environmental conditions. The nutrient scenario in host conditions, especially the human host, is that of a rich source of different carbon sources as well as amino acids that may also serve as carbon sources. As part of a bachelor thesis (by Philipp Demling), phenazine production by strain PA14 was analysed in host-like or nutrient-rich conditions. This section sought to understand whether the presence of the fermentation product 2,3-BD stimulates a change in virulence factor production under these nutrient-rich conditions (similar to those observed above; in this Chapter and Chapter 3). Here, the SCFM medium, which is a formulation of the nutrient composition of the cystic fibrosis lung, was used (48). The
composition provides for the addition of glucose to a final concentration of 3.2 mM. PA14 was grown in medium containing 3.2 mM glucose and in a another SCFM medium where glucose was exchanged for 2,3-BD. Since growth of *P. aeruginosa* in cystic fibrosis mucus largely grows under microaerobic to anaerobic conditions, cultures were grown under microaerobic conditions (125).

In medium provided with 2,3-BD, PA14 grew to a higher optical density compared to glucose (1.8 and 2.8 for glucose and 2,3-BD, respectively. The PA14 cultures provided with 2,3-BD produced four-times more PYO compared to the SCFM cultures supplemented with glucose (Figure 5.17). The PCA amounts produced with the two substrates by the two cultures (glucose and 2,3-BD) were similar. These PCA concentrations were generally lower than those recorded for cultures under nutrient limited conditions (Table 5.1, Table 5.2).

Pyoverdine levels were not different with glucose or 2,3-BD as substrate. As already mentioned, the levels of pyoverdine may be influenced by the levels of iron supplied. Therefore, with increasing growth and subsequent iron consumption, iron may be limited triggering the need to produce more siderophores. However, enhanced growth with 2,3-BD did not stimulate pyoverdine production; indicating a sufficient supply of iron (Figure 5.17).
Figure 5.17: Growth, phenazine and pyoverdine production in SCFM medium with, additional, glucose or 2,3-BD.
Left: The three bars represent sampling times; 11, 24 and 52 hours, respectively. Right: Growth measured as OD600 from three biological replicates. Reduced aggregates and biofilm formation was observed; therefore, OD was considered sufficient as a measure of growth.

5.5 Discussion

5.5.1 Substrate consumption and biomass generation

In host and environmental niches, the different available carbon sources such as fermentation products or carbohydrates may be available for utilisation by *P. aeruginosa*. This may stimulate different regulation mechanisms that ensure efficient utilisation of the available resources as well as confer fitness in nutrient acquisition in competitive niches. For instance, the catabolite repression mechanism has also previously already been linked to virulence (108). Therefore, co-fed cultures of glucose/2,3-BD where two carbon sources stimulate the cells in parallel were also tested. These cultures exhibited a diauxic growth whereby glucose was depleted before 2,3-BD
was taken up, which confirmed the behaviour of co-fed cultures from experiments under the influence of the electrode (Chapter 3-section 3.3.3).

In this comparison, two additional carbon sources with properties that would provide more insight into nutrient-stimulated virulence were included; succinate: a simple substrate preferred by \textit{P. aeruginosa} and octanoate: a substrate available under host conditions, whose metabolisation influences virulence (101, 122, 126). The preferred carbon source (succinate) did not mediate the generation of the highest biomass amounts due to, most likely, an increase in media pH (Figure 5.8). Instead, octanoate mediated the production of the highest biomass amount and growth rate (Figure 5.8, Figure 5.14). Overall, this data suggests that the generation of biomass was influenced by the carbon source available. However, since octanoate provided more carbon and energy, the influence of the carbon source is most likely related to the amount of energy gained from the substrate. Also, since the strain KRP1 seemed to generate, averagely more biomass compared to PA14, the influence of the carbon source on biomass generation also depended on the strain.

The carbon source uptake rates together with the energy density are important aspects in virulence factors production. Cellular availability of the carbon source for energy generation may influence the perception of nutrient limitation (or energy levels) and, therefore, the decision to switch on virulence factors secretion. The production of virulence factors, for instance PYO, was early shown to be influenced by the cellular ATP levels (127).
5.5.2 The carbon source determines the level of virulence generation in minimal medium

The data presented in this chapter suggests that 2,3-BD may stimulate phenazine production and phenazine spectrum depending on conditions available, and this stimulation might influence the strains differentially. This is in line with earlier reports made in AB medium; which was also used in this study (52). In that study, it was observed that 2,3-BD, produced by a glucose-fermenting partner organism, mediated a shift in phenazine spectrum from the production of increased 1-HP to increased production of PYO, and overall more phenazine production. In the current study, however, 1-HP was below detection limit. This cooperative behaviour might be a picture of the interactions of fermenters with *P. aeruginosa* in polymicrobial infections or environmental conditions where they share niches (51, 53). The microorganisms, ultimately, form, energetically, beneficial synergistic interactions (53).

An outstanding high production of PYO was observed in cultures grown with octanoate as the sole carbon source under aerobic conditions for strain PA14. Fatty acid degradation has been shown to be interconnected with virulence generation in a number of species including *P. aeruginosa* (121, 128, 129). However, this exceptional stimulation of PYO production by octanoate seems to be strain specific. It was also observed that in the same culture relatively more PCA was detected; hence, under this condition octanoate consumption leads to, overall, increased phenazine production (considering the detected PCA and PYO). Whether this phenomenon is a related to detection of octanoate as a cue implying host residence and therefore the need for PYO, or is an electron discharge strategy, is an interesting question to be answered. *In vivo* and *in vitro* loss of pathogenicity fitness in *P. aeruginosa* due to a mutation in *fad* genes responsible for metabolism of fatty acids has been reported (121). Mutants that could not metabolise fatty acids showed altered swarming, production of proteases, rhamnolipids and lipases. Even though
phenazines were not considered in that report, it is expected that that these mutant would display altered phenazine production, since this virulence factors are all, partly, controlled by the QS (121).

Phenazine production in cultures provided with succinate was, generally, low except for PA14 under aerobic conditions (Table 5.2). The growth of succinate was, however, limited by increase in pH, and this must have influenced phenazine production (47). Reduced growth has been mentioned as a trigger of PYO production (127). Hence, the increased PYO per CDW production for PA14 under aerobic conditions might have resulted from limited growth. Further, for succinate, the catabolite repression through the CRC proteins downregulates the production of PYO. This was demonstrated in the overproduction of PYO by mutants lacking the $ crc $ genes (110).

Pyoverdine is essential for iron acquisition and virulence generation of $ P. aeruginosa $ is impaired in absence of pyoverdine (130). Under aerobic conditions, $ P. aeruginosa $, like many microorganisms, may be iron limited due to occurrence of iron in its insoluble form as Fe-III (131). The two strains considered produced comparable levels of pyoverdines. They produced relatively higher pyoverdine concentration with glucose as the carbon source. Additionally, PA14 produced elevated pyoverdine levels with octanoate. In $ P. putida $, it was also observed that pyoverdine production was influenced by the carbon source; the highest pyoverdine amount was measured with citrate followed by acetate, succinate and lastly glucose (132). In another report, amino acids were found to influence the production of pyoverdines in $ P. syringae $ pv under Fe-limited conditions (133).
For rhamnolipids, indications of the influence of the carbon source were also observed. On average increased rhamnolipid production was observed with octanoate. However, the pattern of rhamnolipid production between glucose and 2,3-BD was not clear. Major differences in the levels of production among the strains were also not clearly discernible. Increased rhamnolipid production with glucose as the carbon source together with sodium nitrate as the nitrogen source was reported for *P. nitroreducens* (134). In another carbon source comparative study, cashew apple juice was reported as the suitable carbon source with which *P. florescens* produced the high rhamnolipid yields. Cashew nuts contain fatty acids and, probably, the observed rhamnolipid production with octanoate might be as result of the effect of the fatty acids (135).

Data presented here shows, to an extent, a dependence of motility on the carbon source. Presumably, according to Schrout *et al.* (2006), our results suggest that with succinate (with which the highest swarming was observed\(^2\)) a flat less stacked biofilm will be formed (45). The carbon sources octanoate, 2,3-BD and glucose, which exhibited reduced swarming will form aggregates or stacked biofilm. A carbon source dependent swarming activity has been reported and succinate was observed to mediate the highest activity (45). This swarming is however subject to the nitrogen source combined with the carbon source. It was observed that succinate does not induce this increased swarming if combined with aspartate as the carbon source (57). It appears that, with octanoate, both strains are barely motile. This may have far reaching implications in biofilm formation and general virulence generation of *P. aeruginosa*. It should be noted, though, that the nutritional influence on motility is interconnected with the QS system

\(^2\) Increased swarming results in increased surface movement. Hence, the bacteria are disseminated over the surface of attachment resulting in flat biofilm distributed over the entire surface of attachment. When the surface movement is reduced due to reduced swarming activity, cells are less disseminated resulting in aggregates and consequently stacked biofilms (Schrout *et al.* (2006).
The induction of swarming by succinate, for instance, is attenuated in las and rhl mutant (45). The mutation of these two systems was found not to affect swarming induced with glucose and glutamate (45). There is, therefore, an indication of background regulations that impact the outcomes of nutritional influence on biofilm formation. These regulations may be based on different environmental factors and cellular physiological status, and their actions shape the motility patterns. The motility activity observed was also influenced by the strain. Strain KRP1 seems to generally have lower motility activity compared to PA14. It will be interesting to find out whether this is related to the lower QS activation activity observed.

5.5.3 Substrate dependent AHL QS signal concentration

Since the virulence factors production is controlled by the QS system, it was imperative to assess the levels of QS activation with the different carbon sources. The data presented here suggest that, for PA14, 2,3-BD mediates rapid production of the C-4-HSL under aerobic conditions. Under microaerobic conditions, however, the peak concentration of the C4-HSL was higher with glucose. There were no clear correlations between C-4-HSL concentration and the subsequent 3-oxo-C12-HSL as the highest concentration was recorded with the equimolar mix of glucose/2,3-BD. Strain KRP1 also appeared to be less active in 3-oxo-C12-HSL production compared to PA14 and there were no clear patterns between the two aeration conditions considered. Increased production of QS signalling molecules with simple carbon sources in minimal medium as compared to rich medium has been reported (21). It was also noted in that report that the levels of QS activation were not always cell density dependent. In comparison to octanoate, it appears that with glucose and 2,3-BD higher amounts of C-4-HSL are secreted. Therefore, the increased production of especially PYO in octanoate-grown cultures did not correspond with higher accumulation of C4-HSL. This result suggests that increased Las activation does not simply
result in increased phenazines. Similar reports have noted that the increased LasI and RhlI activation does not necessarily result in increased activation of the cognate receptors LasR and RhlR (21, 31). Other regulatory elements interplay to control this activation in line with environmental parameters (31).

5.5.4 Fermentation product 2,3-BD influences production of the virulence factor phenazine by strain PA14 in host environment

To test the possible influence of 2,3-BD in host conditions (nutrient rich conditions), SCFM medium was used, which contains all the nutrients and salts in the concentrations as they are often found in the cystic fibrosis lung sputum (48). Supplementation of the medium with 2,3-BD led to the production of 5-times more PYO compared to medium supplemented with glucose. The levels of PCA in the SCFM medium with glucose remained slightly more than those of the medium supplemented with 2,3-BD implying a reduced conversion of PCA into PYO. Overall, 2,3-BD stimulated the production of more phenazines and increased conversion of PCA to PYO. Unlike under limited conditions, the levels of pyoverdine remained similar. This might have been due to the availability of iron. SCFM medium contained 2 times more iron compared to AB medium.

It seems, therefore, that the presence of 2,3-BD leads to increased growth and more PYO production from PCA. Indeed, butanedione (diacetyl) has been detected in the cystic fibrosis lung and genes of its catabolism were found to be upregulated in P. aeruginosa (51). In that report it was hypothesised that this is a form of synergism in which Streptococcus spp. produces diacetyl, which is taken up by P. aeruginosa and this stimulates it to produce more phenazines. A recent report has shown that P. aeruginosa grown with 2,3-BD possesses increased colonisation fitness
and immune system activation (136). There is, therefore, growing evidence that the presence of 2,3-BD and its related products may increase the virulence of \textit{P. aeruginosa} in the host.

As already postulated, under the lung conditions fermenters may produce 2,3-BD and \textit{P. aeruginosa} produces PYO (and other phenazines) that will provide additional electron acceptors for shuttling electrons to terminal acceptors (51). Such interactions between fermenters and \textit{P. aeruginosa} in the environment have also been observed in BES (53). One of these interactions was described as a synergistic interaction where \textit{P. aeruginosa} consumes the 2,3-BD from the fermenter \textit{E. aerogenes} and the fermenter utilises phenazines for respiration (53). Another possibility might be that 2,3-BD is detected as a cue indicating the presence of competitors, and phenazines (together with other virulence factors which are co-regulated) produced are intend to act as antibiotics. This assumption can be drawn from the observation that \textit{P. aeruginosa} can detect peptidoglycans from gram-positive bacteria and produce virulence factors against them (49). The detection of other metabolites from fermenters or other bacteria and fungi by \textit{P. aeruginosa} has been reported (50, 88). These are important interactions, which can be exploited in the search for remedies and biotechnological applications.

Thus, a number of \textit{in vitro} reports using minimal medium under different conditions have also provided insights into the influence of 2,3-BD (52, 53). Even though growth under such minimal media conditions do not provide the similar conditions as they occur in the cystic fibrosis lung or generally host conditions, they provide knowledge and leads towards the signalling and interactions underlying the influence of 2,3-BD. Data presented in this section of the thesis using the synthetic CF medium (mimicking the conditions that \textit{P. aeruginosa} faces in the cystic lungs), confirms this phenomenon of the 2,3-BD stimulated-virulence factors production in vitro.
5.6 Conclusions

There is growing evidence that nutrition plays important roles in virulence generation in the opportunistic pathogen *P. aeruginosa*. In polymicrobial infections cross feeding of nutrients such as fermentation products in synergistic interactions may lead to more complex regulatory networks that enhance virulence. Here the influence of 2,3-BD on virulence factors production in comparison to other selected carbon sources during growth under aerobic and microaerobic conditions was tested. With the different carbon sources, QS activation levels differ depending on the strain and the carbon source provided. Moreover, the levels of signalling molecules produced do not, in all cases, correspond to increased virulence factors production. The study confirms a strain and prevailing conditions-related influence of 2,3-BD on the production the phenazines in nutrient limited conditions (minimal medium). 2,3-BD also stimulates a shift in the phenazine spectrum to more PYO production in the nutrient rich SCFM medium that mimics the cystic fibrosis lung. Further, the section demonstrates a carbon source dependent motility of *P. aeruginosa*
Chapter Six

The role of 2,3-BD in signalling and virulence stimulation in *Pseudomonas aeruginosa* PA14

6.1 Background

In the previous chapters, it was confirmed that 2,3-BD increased phenazine production and subsequently current generation (Chapter 3-section 3.3.2). This mirrors the synergistic interactions that occur between fermenters and the redox mediator producer (*P. aeruginosa*) in BES (52, 53). To be able to utilise this interaction, a comprehensive understanding of signalling or mechanisms of interactions via metabolites is necessary. In this chapter, the role of the butanoate pathway and that of 2,3-BD in virulence generation was explored.

6.1.1 2,3-BD might influence *P. aeruginosa* via metabolic and/or signalling reactions

In chapter 3, it was confirmed that this metabolite influences phenazine production in a strain specific manner and subsequently modulates electric current generation. This substance is a shared metabolite among fermenters and *P. aeruginosa*, which stimulates the production of phenazines, and the phenazines can be utilized by both the producer and the fermenter (53). In the same way elevated microbial production of the 2,3-BD precursor butanedione (also called diacetyl), which can be spontaneously reduced to 2,3-BD was shown to exacerbate disease outcome in a cystic fibrosis lung (51). This pointed to a synergistic interaction between the producer (thought to be *Staphylococcus aureus*) and the consumer (*P. aeruginosa*) of this fermentation product. In the former studies, it was further shown that during this increased virulence generation, the QS system was upregulated (53). Hence, the conclusion that 2,3-BD exerts its influence via QS was imperative. It is however not clear how this fermentation product mediates virulence generation and which signalling cascades are involved. Understanding the
mechanism of signalling and signal transduction of this fermentation product will be beneficial in not only developing control remedies but also technologically in optimising this synergistic interaction for electric current generation in BES.

*P. aeruginosa* itself produces acetoin, which is the intermediate that is reduced into 2,3-BD during growth on glucose. This implies that the butanoate pathway is switched on during growth on glucose (Chapter 3-section 3.3.1). The role and importance of switching on this pathway in *P. aeruginosa* is not clear. In fermenting bacteria such as *Enterobacter*, it is known that 2,3-BD production plays important roles of regulating pH to avoid acidification, and in balancing the NADH-NAD+ ratio. It may also provide a source of reducing equivalents when the carbon source has been depleted (89). A possible explanation for switching on this pathway in *P. aeruginosa* is that the 2,3-BD produced (and other related metabolites; acetoin and diacetyl) provide a similar stimulatory effect for virulence factors production like the exogenous 2,3-BD in a possible self-stimulation loop. Related reports of the role of the butanoate pathway in virulence generation are, for example, that of *Pectobacterium carotovorum* (137). Mutants that were not able to produce 2,3-BD were not able to propagate disease on potatoes under anaerobic conditions. Here, it was hypothesised that this pathway provides alkaline conditions that are necessary for lyases. A number of bacteria and fungus have also been shown to activate this pathway for optimal activity of their lyases (137-139). I hypothesize here that the acetoin or probably the 2,3-BD and diacetyl that is produced by *P. aeruginosa* intrinsically may exert a similar influence on virulence factor regulation as the exogenous substances provided by fermenting partner organisms.

*P. aeruginosa* is known to maintain a stable NADH/NAD+ ratio in the absence of an electron acceptor via the production and utilization of phenazine redox mediators (113). Since 2,3-BD
catabolic metabolism provides additional reducing equivalents it is presumable that *P. aeruginosa* may respond by producing more electron shuttles (this remains to be experimentally confirmed). Also, considering that 2,3-BD has a higher degree of reduction in comparison to, for instance glucose, its oxidation yields more reducing equivalents; and this may necessitate the production of more redox mediators such as phenazines. It is, therefore, not clear whether the increase in phenazines production is due to the need to balance the reducing equivalents provided by the consumption pathway of 2,3-BD. However, some of the reports indicate an overall increase in the generation of other virulence factors other than phenazines; implying a possible signalling function of 2,3-BD to stimulate broader virulence (52). This study sought to determine whether 2,3-BD is indeed a signal that activates the QS system, or the physiological consequence of its catabolism, in terms of the amount of reducing equivalents, which necessitate the production of this virulence factor. Either way, it is imperative to experimentally determine the connection of 2,3-BD to the central QS system and (or) virulence generation (or phenazine production).

### 6.2 Experimental set up

To obtain clear results from the addition of external 2,3-BD to a *P. aeruginosa* culture, mutants of the butanoate pathway, which leads to the *in-vivo* formation of acetoin and 2,3-BD were generated in strain PA14. The genes for all predicted acetolactate synthase functions, *als, ilvI* and *ilvG*, that condense two pyruvate molecules to acetolactate were knocked out as outlined in chapter 2-section-2.4 (Figure 6.1). To determine whether 2,3-BD is indeed a signal that activates the QS, it was also necessary to generate mutants that cannot oxidise it for catabolic usage. The *aco* gene cluster (*acoXABCadh*) responsible for the oxidation of 2,3-BD was knocked out as
Figure 6.1: Schematic representation of the *P. aeruginosa* butanoate pathway. The red cross indicates the point at which knock out mutants were generated. The following genes were knocked out: *als*-acetolactate synthase, *ilvI*-putative phosphonopyruvate decarboxylase and *ilvG*-putative acetolactate decarboxylase. These are the enzymes that can possibly convert pyruvate to acetolactate.

Figure 6.2: Schematic representation of the 2,3-butanediol catabolism pathway. A-represents the gene cluster responsible for 2,3-butanediol catabolism and B-describes the reactions performed by the involved enzymes in 2,3-butanediol oxidation outlined in chapter 2-section 2.4 (Figure 6.2-A). Primers for generating these knockouts are listed in Table 9.2 (Appendix 2).

PA14 mutants unable to produce 2,3-BD and acetoin were grown in AB medium provided with glucose and 2,3-BD as carbon sources. The mutant lacking both the butanoate and the 2,3-BD catabolism pathway was cultured in media containing a co-feed of glucose and 2,3-BD. Since the interruption of the butanoate pathway affects the synthesis of the amino acids leucine, valine and
the vitamin pantothenate, all cultures were provided with those amino acids. Their growth, carbon source metabolism and production of phenazines were evaluated. This evaluation was performed in AB medium in order to decipher the role of 2,3-BD and the butanoate pathway as it was observed in chapter 3. However, little (in some cases no) growth was observed for the mutant cultures. After it was confirmed that a strong pH drop adversely affected the growth of these cultures, an experiment was conducted in a MOPS-buffered medium for confirmation.

6.3 Results

6.3.1 Does 2,3-BD act as a signal for virulence generation?

6.3.1.1 Growth and carbon source metabolisation of the generated mutants

Growth of the acetolactate synthase mutants was compared to that of the wild type (these genes are responsible for synthesis of the precursor molecule for the butanoate pathway). The \( \Delta als, ilvI, ilvG \) triple mutant did not grow in AB medium containing glucose, but showed delayed and strongly reduced growth with 2,3-BD as substrate. With the supply of the amino acids and pantothenic acid, whose synthesis was interfered in the mutant, it was anticipated that the mutant would grow on glucose under these conditions (Figure 6.3). It was noted that the low pH after 8 hours must have limited its growth.
The wild type consumed glucose and ketogluconate and traces of acetate were detected in the secreted metabolites (Figure 6.4). Even though it did not grow, the $\Delta als, ilvI, ilvG$ triple mutant also consumed glucose converting it into majorly ketogluconate under both aeration conditions. However, the ketogluconate was not further metabolized. The accumulation of this acid likely is also the reason for the low pH in this cultivation. It was not clear why the further metabolism of ketogluconate was impaired. In cultures provided with 2,3-BD, there was no adverse pH change. However, the mutant also barely grew and converted 2,3-BD into acetoin, which it did not re-consume (76 hours; Figure 6.4).
From the data above, it was clear that the acetolactate mutant did not grow as expected and further comparisons were not possible under the experimental conditions provided. Since it was not clear why the further metabolism of ketogluconate is hampered and pH was thought to have dropped to unfavourable levels, an attempt to control pH was made. MOPS medium with a stronger buffering capacity was used to confirm the ability of the mutants to grow. The medium was prepared as outlined in chapter 2-section 2.2 and supplemented with glucose. Here, the acetolactate mutants grew to a similar final OD but at a somewhat slower rate compared to the
wild type. A pH drop was observed after 4 hours, which reached its maximum drop after 16 hours. The pH recovered after 24 hours and by the end of the experiment it was back to the starting pH of 6.37. The pH recovery coincides with the phase of rapid increase in growth; hence, it can be inferred that the initial delay in growth might have been caused by the low pH. The wild type cultures also showed a slight drop in pH, which recovered rapidly (after 16 hours; Figure 6.5).

Both the wild type and acetolactate synthase mutant consumed glucose at the same rates and produced ketogluconate, acetoin and acetate as the metabolites. The wild type produced more ketogluconate (up to 21 mM and 18 mM for aerobic and microaerobic conditions, respectively) compared to the acetolactate synthase mutant (6.9 mM and 9.2 mM or aerobic and microaerobic respectively; Figure 6.6). It was confirmed here that the mutant was capable of growing but the reason associated with its growth in MOPS and not in AB medium still remains to be confirmed. Unfortunately, it was not possible in the given time to also evaluate the acetolactate mutant in MOPS medium with 2,3-BD.

![Figure 6.5: Growth and pH change of the wild type and acetolactate synthase mutant in MOPS buffered medium. Dotted lines show cultures grown under microaerobic conditions and straight lines under aerobic conditions.](image)
Further, a mutant of the 2,3-BD catabolism pathway was generated (Figure 6.2). Lack of this pathway was found not to have any physiological impact on growth in AB medium containing glucose. This was demonstrated by the growth of the ΔacoXABCadh, which generated biomass amounts similar to the wild type. Another mutant lacking both the catabolism and the synthesis pathway was generated (ΔacoXABCadh,Δals,ilvI,ilvG). In comparison to ΔacoXABCadh, the mutant lacking the 2,3-BD catabolic pathway and the butanoate pathway, expectedly, grew barely in AB medium with glucose (Figure 6.7-a). The same effect observed with the acetalactate synthase mutant i.e., accumulation of ketogluconate and low pH might have impacted the growth of this mutant (Figure 6.7-c). This mutant also did not grow when provided with a co-feed of glucose and 2,3-BD (Figure 6.7-a), where it again converted glucose to ketogluconate but did not utilize the provided 2,3-BD confirming the lack of the 2,3-BD catabolic pathway.
Figure 6.7: Growth and carbons source uptake of the 2,3-BD catabolic and acetolactate synthase mutants in AB medium. The graphs show (a) biomass for $\Delta$acoXABCadh only and $\Delta$acoXABCadh,Als,ilv1,ilvG, (b) glucose uptake by $\Delta$acoXABC only mutant, (c) glucose uptake by $\Delta$acoXABCadh,Als,ilv1,ilvG and (d) consumption of glucose and 2,3-BD by $\Delta$acoXABC,Als,ilv1,ilvG supplied in a 20 mM glucose+10 mM 2,3-BD co-feed. The metabolites: Keto-gluconate, acetate, acetoin and gluconate are plotted on the left right-axis.

6.3.2 Phenazine and pyoverdine production

The objective here was to understand the possible contribution of the endogenously and exogenously supplied 2,3-BD and the relationship of the butanoate pathway to phenazine and pyoverdine synthesis. This may also provide insight into the role of the butanoate pathway in the phenazine production in relation to energy metabolism. A comparison of virulence factors production of the wild type to a mutant that can neither produce 2,3-BD (and the related metabolites acetoin and diacetyl) nor consume it, may shade light on whether 2,3-BD is indeed a
signal. Mainly the phenazine production of the wild type and that of the butanoate pathway mutants in standard AB medium containing glucose and 2,3-BD was to be compared to understand the mode of action of 2,3-BD observed in chapter 3.

However, since the butanoate pathway mutants did not grow in AB medium containing glucose (hence, no phenazine and pyoverdine production), it was not possible to determine the influence of activating the butanoate pathway on phenazine and possibly pyoverdine production using AB medium. In AB medium containing 2,3-BD, the butanoate pathway mutant grew slightly and produced phenazines only under microaerobic conditions. However, the PCA and pyoverdine concentrations of this mutant were extremely low compared to those of the wild type (Figure 6.8). The mutant lacking the butanoate and the \( acoXABCadh \) gene cluster responsible for the catabolism of 2,3-BD was also grown in AB medium containing 20 mM glucose+10 mM 2,3-BD. The hypothesis here was that, this mutant will not metabolise the provided 2,3-BD but, if it is a signal, it might use it for signalling to produce more phenazines or other virulence factors. However, this is only possible with comparison to the amount produced in the glucose medium (“without the 2,3-BD signal”) and since there was no growth under this condition, this hypothesis could not be verified.
As already noted, MOPS medium was used to confirm the viability of the butanoate pathway mutant (Figure 6.5). In MOPS medium some comparisons were possible but data are only available with glucose as a substrate, since this experiment was performed at the very end of this thesis work. Here, with glucose as the carbon source, differences were observed concerning the time and concentration of PCA, PYO and Pyv for the butanoate pathway mutants and the wild type (Figure 6.9). The maximum phenazine and pyoverdine concentrations were calculated for comparison. The mutant produced more PCA and pyoverdine per OD compared to the wild type. PYO production was low for both cultures, but slightly higher for the wildtype (Figure 6.10).
Figure 6.9: Phenazines and pyoverdine production by the wild type and butanoate pathway mutant grown in MOPS medium provided with glucose (also see Figure 6.5). From left to right are graphs of the wild type and mutant, respectively. Dotted lines indicate microaerobic conditions aerobic conditions. Pyoverdine is plotted on the right axis and phenazines on the left.

Figure 6.10: Maximum phenazine and pyoverdine concentration corrected per OD.

From the MOPS medium confirmation, there is an indication of differences in PCA production of the butanoate pathway mutants with glucose as the substrate. Unfortunately, the equivalent experiment and data for 2,3-BD in MOPS medium are not available at this stage. Therefore, in future a more comprehensive comparison of the 2,3-BD catabolism and butanoate pathway mutants under well controlled conditions (pH and oxygen and other environmental factors that
influence the QS) will provide further understanding of the role of the butanoate pathway and comprehensively answer the question of 2,3-BD being a signal.

6.4 Discussion

6.4.1 The butanoate pathway is necessary for growth with glucose

*P. aeruginosa* produces 2,3-BD, acetoin (3-hydroxy-2-butanoic) and diacetyl (butanedione) via the butanoate pathway. Whether the 2,3-BD and related compounds produced *via* this pathway may result in a similar stimulation effect as the exogenously added 2,3-BD is not yet clear. In this chapter, the main objective was to determine whether 2,3-BD is indeed a signal that directly activates the QS system and subsequently phenazine production and if intrinsically produced butanoate pathway products have a similar stimulation effect. Hence, mutants of the butanoate pathway were first generated to ensure that 2,3-BD is not endogenously produced.

However, deleting the butanoate pathway resulted in no growth in the standard AB medium. Only a conversion of glucose into ketogluconate was observed. This resulted in acidification of the medium. Therefore, meaningful comparisons in this medium could not be obtained. Deleting this pathway, therefore, seems to affect glucose metabolism; hence, no growth. It was anticipated that valine, leucine and panthothenate will not be synthesised since they are products of acetolactate. Even though the amino acids (valine and leucine) and panthothenate were provided, the possible explanation for the lack of growth might be the unavailability of those compounds for metabolism. It is possible that isoleucine was also required as was observed in acetolactate mutant of *Corynebacterium glutamicum* (140). It is also known that valine inhibits the growth of wild type *P. aeruginosa* by blocking the activity and accumulation of acetolactate synthase, and the only way to rescue this inhibition is by adding isoleucine (141). Further experiments with the
provision of this amino acid and evaluation of the physiological drawbacks of the lack of butanoate pathway will shed more light on this.

To confirm that pH indeed limited the growth of the butanoate pathway mutants, the mutants were grown in MOPS medium, which provides more buffering capacity. Like for AB medium, the pH dropped in the MOPS medium and limited early growth. However, the recovery of the pH allowed further growth of the mutants. It appears that the mutant grows in MOPS-buffered medium even with only the provision of leucine, valine and panthothenate (without isoleucine as noted above). It is not clear which component or regulation in MOPS medium enables this growth. Moreover, phenazines were detected and quantified. Comparing the wild type (also grown in MOPS buffered medium with glucose), the mutant produced two times lower PCA concentration. The question of the role of the butanoate pathway in virulence factors generation or metabolism-related phenazines production stimulation requires more investigations using different approaches or even media. This also applies to the question whether the intrinsic 2,3-BD and the related products are indeed signal for self-stimulation like the exogenous 2,3-BD.

6.5 Conclusion

_P. aeruginosa_ has been shown to elevate its virulence factor production in medium containing 2,3-BD. In this chapter, an attempt to determine whether this fermentation product is a signal that possibly interacts with the central QS signalling system to activate virulence generation, and the specific two-component system it interacts with was made. 2,3-BD synthesis pathway and catabolism pathway mutants were successfully created. In standard AB medium, metabolism of the mutants was impaired due to probably the interference of the amino acid synthesis. In alternate MOPS medium, there indications that the butanoate pathway plays a role in the
virulence factor production; however, a further determination of its specific role and that of exogenous 2,3-BD requires a strongly buffered medium, appropriate amino acid provision and strictly controlled experimental conditions.
Chapter Seven

The two-component systems RcsC/RcsB and PvrS/PvrR are involved in the perception and regulation of 2,3 BD-stimulated virulence factors production

There are indications that the influence of 2,3-BD includes a change in the QS system of *P. aeruginosa*. Most likely, when 2,3-BD acts a signal to the cell, it relays its influence *via* the QS, which further regulates the genes responsible for virulence factor production. This hypothesis remains to be experimentally confirmed. Some other nutritional elements have also been shown to influence virulence generation and in some cases the activation of the QS has been linked to virulence (48, 50, 88). Like many other environmental factors that influence QS, nutritional cues might be conveying their signalling *via* the two-component systems to the QS. These primary two component systems may convey the specific signal to global two-component systems such as GacS. Alternatively, they may convey their signal to the QS system or directly to the regulators involved.

I hypothesised that signalling with 2,3-BD, being an environmental factor, most likely signals *via* primary two-component systems, which relay the signals directly to the QS system or indirectly *via* global two-component systems. For instance, considering the hypothesis that the intrinsic 2,3-BD might mediate a self-stimulatory loop, it has been reported for *Serratia plymuthica* that QS actually regulates the activation of the butanoate pathway responsible for the synthesis of 2,3-BD (142). This study sheds some light on the possible connection of the butanoate pathway to the QS system. It will be interesting to find out whether the same connection exists for sensing the presence of 2,3-BD in the growth environment leading to activation of the signalling cascade. More specifically, concerning the possible role of the two-component systems, it was shown that...
in *Pseudomonas chloraphis*, GacS plays an important role in activating the production of 2,3-BD. This study also indicates a connection of the two-component system to this pathway even though there is no confirmed connection to sensing of exogenous 2,3-BD. It is, however, likely that in such cases feedback loops exist for stimulation and downregulation, depending on the cellular and environmental status of the substance in question. Even though it is not known whether intrinsic 2,3-BD has an effect of self-stimulation in *P. chloraphis*, that study observed that the 2,3-BD produced elicits systemic resistance to leaf pathogens in tobacco; from which we can probably draw some similarities (143). These reports do not give an implication of a similar scenario for exogenous 2,3-BD, but they at least indicate a complex regulation involving two-component systems and the QS.

In plants, the possible route of 2,3-BD signalling has been reported. In *Arabidopsis*, 2,3-BD produced by *Bacillus* was shown to signal via two-component systems, which are also responsible for the perception of ethylene and cytokinin. 2,3-BD and other volatiles induce systemic resistance against pathogens via these perception systems. Mutants lacking these two-component systems were not able to generate resistance when treated with the *Bacillus* volatiles (137, 144, 145). I raised the question whether there may be similarities in these plant systems with the bacterial systems that may help us identify the possible signalling path of 2,3-BD in *P. aeruginosa*. In this section, an answer to the question whether 2,3-BD signals via a two-component system to activate pathways responsible for virulence generation was sought.

### 7.1 Experimental set up

To evaluate the role of two component systems in 2,3-BD signalling, a comparison of the 2,3-BD signalling in *Arabidopsis* was used to find any similarities with *P. aeruginosa*. As already
mentioned, in *Arabidopsis*, it was observed that 2,3-BD signals via ethylene and cytokinin sensing two-component systems. Ethylene and cytokinin signal through two-component systems to control growth and immunity in plants (146, 147). For cytokinin, the cytokinin response 1 (*cre1*), which is responsible for the synthesis of a hybrid kinase, which in turn contains a receiver for cytokinin, was used in the comparison. For ethylene, the *ein2* genes coding for a hybrid kinase were used in the comparison. Ethylene and cytokinin can potentially signal also through other two-component systems; CRE1 and EIN2 were selected because of their involvement in signalling with 2,3-BD volatiles (145). Results of the comparison were used to identify possible two component systems in *P. aeruginosa* that may be involved in 2,3-BD signalling. Mutants of the identified two component systems in *P. aeruginosa* were generated for further physiological characterisation.

7.2 Results

7.2.1 Signalling of volatile compounds via the plant ethylene and cytokinin systems: are there similarities with *P. aeruginosa*?

The fermentation product 2,3-BD and other volatiles produced by rhizobacteria have been shown to influence defence responses in *Arabidopsis* by signalling via the ethylene and cytokinin two-component perception systems. Ethylene and cytokinin signal via two-component systems to promote growth. Ethylene perception occurs through several hybrid kinases termed ETR2 and histidine kinases termed EIN4. Cytokinin on the other hand is perceived through the hybrid kinases AHK2, AHK3 and CRE1 (146). Mutants lacking the genes (*ein2* and *Cre1*) coding for the EIN2 and CRE1 kinase were shown to lack response to the volatiles such as 2,3-BD produced by *Bacillus* (144, 145). Most likely, this is part of the environmental sensing of *Arabidopsis* conveying signals of the microbial community metabolites. *P. aeruginosa* also responds to 2,3-
BD produced by fermenters in their environmental niches (52, 53). The question arises whether there might be any relationships between the environmental sensing systems of *Arapidopsis* with those of bacteria (especially the two component systems).

*P. aeruginosa* uses two-component systems to sense many environmental conditions including nutrients (reviewed in (148)). It was anticipated that some genetic similarities with the plant ethylene or cytokinin system could be identified to indicate a possible *P. aeruginosa* two-component system activated by 2,3-BD. A degenerated genetic blast of the sensor kinases *ein2* and *Cre1* was performed against the *P. aeruginosa* genome. The similarities revealed that *ein2* (NCBI accession number-NC_003076.8) had similarities with the manganese transporter (*mntH1*; 34% identity) of *Pseudomonads*. Direct similarities of *ein2* with strain PA14 were not found. This result was not considered for further analysis as it was not a two-component system as anticipated. Results for *cre1* (NCBI accession number-NC_003071.7) revealed similarities with a number of two-component system kinases of *Pseudomonads*. For strain PA14, 8 hits were found. The identities of the hits with the whole protein as well as the histidine kinase A (HisKA) region of the protein are listed in table 7.1. HisKA is the point of perception of stimuli and after autophosphorylation, the signal is phosphor-relayed to the regulator. The two-component systems whose similarities were located at the HisKA are, therefore, of interest.
Table 7.1: Similarities of *P. aeruginosa* two component systems with CRE1.

<table>
<thead>
<tr>
<th>Description</th>
<th>Amino acid identity (%)</th>
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<tr>
<td></td>
<td>CRE1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hypothetical protein CIA 04075</td>
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<tr>
<td>Two-component system sensor/response regulator hybrid-rcsC</td>
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<tr>
<td>Signal transduction histidine kinase</td>
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</tr>
<tr>
<td>Chemotaxis-CheY</td>
<td>37</td>
</tr>
<tr>
<td>Kinase sensor protein of two-component regulatory system-pvrR</td>
<td>31</td>
</tr>
<tr>
<td>RcsC</td>
<td>29</td>
</tr>
<tr>
<td>Two-component system sensor</td>
<td>24</td>
</tr>
</tbody>
</table>

<sup>a</sup>-represents overall identity to the CRE protein  
<sup>b</sup>-represents identity to the histidine sensor region; dashes represent cases of no identity to the sensor region, instead the sequence coverage was mainly identical to the receiver domain.

The two-component system sensor kinases Rcs and Pvr showed the highest similarity at the HisKA region. Rcs and Pvr were found to be located in the PAPI virulence island (149). It was hypothesised, therefore, that either the Rcs or Pvr sensing systems play a role in 2,3-BD signal transduction similar to the signalling that occurs in *Arapidopsis*. These systems are composed of the sensor kinases RcsC and PvrS and the regulators RcsB and PvrR (148). Both systems RcsC/RcsB and PvrS/PvrR regulate the *cup* genes expression antagonistically. Expression of *cupD* leads to the formation of fimbriae that are important in biofilm formation (150). Mutants lacking the RcsC/RcsB and PvrS/PvrR systems have been shown to possess attenuated virulence (149). To determine whether these two-component systems play any role in 2,3-BD signalling, a knock-out mutant of the RcsC/RcsB and PvrS/PvrR two-component systems was generated as outline in chapter two (Chapter 2-section 2.4). Regions flanking the four genes of interest were amplified using the primers provided in Table 9.3 (Appendix 2). Their growth in medium
containing glucose, 2,3-BD, and octanoate, and phenazine/siderophore production were determined.

7.2.1.1 Growth and carbon source metabolisation of the RcsC/RcsB and PvrS/PvrR two-component systems mutants

The RcsC/RcsB and PvrS/PvrR two-component system double mutant was cultured under aerobic conditions in AB medium provided with glucose, 2,3-BD, and octanoate as the substrates. The mutant generated biomass levels that were comparable to those of the wild type during growth in medium containing glucose and 2,3-BD (1.3 and 0.9 g CDW L\(^{-1}\) for glucose and 2,3-BD, respectively for the wild type and 1.2 and 1.0 g CDW L\(^{-1}\) for glucose and 2,3-BD, respectively for the mutant). In medium containing octanoate as the carbon source, the mutant generated twice the amount of biomass generated by the wild type (1.2 and 2.4 g CDW L\(^{-1}\) for the wild type and mutant, respectively; Figure 7.1).
Figure 7.1: Growth of the Rcs and Pvr two-component system mutant provided with different substrates.
(a) Biomass produced in medium supplied with glucose, 2,3-BD and octanoate, (b) glucose uptake, (c) 2,3-BD uptake and (d) octanoate uptake. For octanoate no secreted metabolites were detected. All cultures were grown in triplicates under aerobic conditions.

Glucose was taken up rapidly and depleted after 16 hours of growth. 2,3-BD was depleted after 48 hours of growth. Similar to glucose, octanoate was also taken up rapidly and depleted after 16 hours of growth (Figure 7.1-b, d and e).
7.2.1.1 Phenazine and pyoverdine production by the RcsC/RcsB and PvrS/PvrR two-component systems mutant

To assess the role of the RcsC/RcsB and PvrS/PvrR two-component systems in signalling with 2,3-BD, the virulence factors phenazines and pyoverdine were quantified and compared to the wild type behaviour. The levels of PCA produced by the mutant grown in medium containing glucose, 2,3-BD, and octanoate were below detection limits. It was noted that in medium containing glucose a compound with an absorbance spectrum similar to PCA was accumulated (Appendix 4). Elevated amounts of PYO were detected in cultures provided with glucose as the carbon source compared to both 2,3-BD and octanoate. In comparison to the wild type, the amount of PYO produced by the mutant in medium containing glucose was over 10-times higher. Conversely, the mutant produced over 10-times lower amounts of PYO compared to the wild type in medium containing octanoate as the carbon source. Considering the phenazine spectrum, the RcsC/RcsB and PvrS/PvrR system mutants produced, additionally, PCN in 2,3-BD and glucose medium and 1-HP in medium containing 2,3-BD. Overall, the deletion of these two component systems drastically affects the amounts and spectrum of the phenazines, and this effect seems to be different among the carbon sources (Figure 7.2-b, d, d). Moreover, it attenuates the virulence factor stimulation effect of 2,3-BD.
Figure 7.2: Phenazine and pyoverdine production by the RcsC/RcsB and PvrS/PvrR two-component system mutant (for comparison with the wild type also see Figure 9.1 and Figure 9.2).

The graphs represent phenazine production by the mutant in medium containing; (a) glucose, (b) 2,3-BD and (c) octanoate. Graph d shows a comparison of maximum amounts of phenazines produced by the wild type (WT) and the mutant (Mut) in medium containing glucose, 2,3-BD and octanoate. Data is presented for triplicate cultures grown under aerobic conditions. *-represents a compound that was not quantified (see Appendix 4).

Pyoverdine production was similar between the wild type and the mutant grown in medium containing 2,3-BD. The mutant grown with glucose or octanoate produced 2-times the concentration of pyoverdine produced by the wild type. It should be noted, however, that the mutant produced two times more biomass compared to the wild type with octanoate. This might have influenced the need for more iron and hence more siderophore production. Since the
pyoverdine production remains constant in medium containing 2,3-BD, it can be inferred that the mutation does not influence pyoverdine production with 2,3-BD (Figure 7.2-b, c, d).

7.3 Discussion

7.3.1 The two-component systems RcsC/RcsB and PvrS/PvrR in the PAPI virulence island are involved in the regulation of phenazine production

By comparing the genetic foundation of the two-component systems responsible for the perception of cytokinin and ethylene in plants with *P. aeruginosa*, the two-component systems RcsC/RcsB and PvrS/PvrR were found to be the closest in similarity. Among the 8 two-component systems that were found to have sequence similarities with the cytokinin sensor kinase (CRE1), four have similarities at the HisKA region and RcsC was the most identical (62%). Both systems occur adjacent to each other in the PAPI Pathogenicity Island and, since it was not clear which one is specifically important, both were deleted. Deletion of these systems leads to a shift in the phenazine amount and spectrum (Figure 7.2-d). PYO production is increased with glucose as the carbon source, and decreased in medium containing octanoate. Therefore, the role of these systems in PYO production seems to be carbon source dependent. On the other hand, deletion of these systems seems to generally lead to reduced PCA production. Mostly likely, the PCA produced is efficiently converted to other phenazines and no secreted PCA is detected in the medium. An unidentified substance with an absorption spectrum closely similar to that of PCA accumulates in medium containing glucose (Appendix 4). This is probably an alternative molecule produced instead of PCA or a precursor of PCA that is not converted to PCA. Averagely, it appears therefore, that the deletion of RcsC/RcsB and PvrS/PvrR leads to increased production of phenazines in cultures supplied with glucose and reduced production in cultures supplied with 2,3-BD and octanoate (Figure 7.2-b, c and d).
The data here suggest that these two-component systems are involved in the regulation of phenazine production differently depending on the carbon source: while PYO production is stimulated with 2,3-BD and octanoate, but not with glucose, in the wild type, it is now stimulated with glucose, but not in cultures grown with 2,3-BD or octanoate in the sensing mutant. The data presented was obtained under aerobic conditions. It is likely that under oxygen limited conditions the phenomenon described here will be influenced also by oxygen availability. Hence, it will be necessary to get insight into the regulation of these systems under controlled microaerobic conditions.

The RcsC/RcsB and PvrS/PvrR two-component systems have been shown to control biofilm formation by controlling the expression of cupD genes that are responsible for the formation of Cup fimbriae (150). Cup fimbriae perform different functions of attachment, which promote biofilm formation. The function of biofilm formation is indeed under the influence of the QS system; the same case as the phenazine production. It is also important to note that phenazines are produced, especially, in the biofilms and during biofilm dispersal (reviewed in (59, 94, 113)). It will be interesting to find out the intricate interrelation and probably co-regulation via these two-component systems. The RcsC/RcsB and PvrS/PvrR systems regulate the cupD expression antagonistically, whereby, RcsC/RcsB is an activator and PvrS/PvrR a repressor (151). This raises the question whether the same is the case with phenazine production; whether the converse production of phenazine results from the different interactions of carbon sources with these two-component systems. This question should be further followed with mutants lacking one of the systems and grown in medium containing these different carbon sources.
Overall, the data presented here suggest a previously unknown profound role of the RcsC/RcsB and PvrS/PvrR two-component systems located on the PAPI Pathogenicity Island in regulating phenazine production. The fermentation product 2,3-BD and seemingly other available carbon sources influence phenazine production via these two-component systems. Further investigations into the interaction of these two-component systems with phenazine production may provide a comprehensive overview of the intricate mechanisms and interaction that interplay in this nutritionally-stimulated virulence factor production.

7.4 Conclusion

Arabidopsis two-component systems used in the perception of 2,3-BD shows similarities to P. aeruginosa systems including the RcsC/RcsB and PvrS/PvrR found in the PAPI pathogenicity island. These systems are involved in carbon source-related control of the production of phenazines. For 2,3-BD, the RcsC/RcsB and PvrS/PvrR systems downregulates the overall phenazine production. Further, lack of these systems leads to a shift in the phenazine spectrum depending on the available carbon source. Findings in this chapter have far reaching implications in understanding the P. aeruginosa polymicrobial ecology and mechanisms of interspecies interactions.
Chapter Eight

Summary, conclusions and outlook

*P. aeruginosa*, one of the quintessential pathogens, produces a wide array of virulence factors to propagate pathogenicity. Some of these factors play different roles in conferring *P. aeruginosa* outstanding fitness in different ecological niches. In microbial consortia, some of the virulence factors are employed in competition and other forms of interaction. Phenazines, for instance, act as antimicrobials and have been shown to be used as redox mediators in synergistic interactions. Metabolite-transfer based synergistic interactions with 2,3-BD as the shared metabolite between *P. aeruginosa* and *E. aerogenes* have been described (52, 53). The phenazines produced here confer *E. aerogenes* with electroactivity; enabling it to respire with an electrode. Therefore, this potential of *P. aeruginosa* can be exploited in BES to enhance electron transfer. This study sought to provide deeper understanding into the phenazine production and current generation in BES. Further, it sought to provide insight into the regulation and signalling via 2,3-BD that leads to the production of phenazine and other virulence factors.

In chapter three, the phenazine redox mediator production of three selected *P. aeruginosa* strains with different carbon sources was determined. Here, the suitability of the different strains for application as redox mediator in BES was assessed. The environmental isolate KRP1 was found to be, averagely, most electroactive under the conditions that were tested. It produces large amounts of PCA for electron shuttling compared to strain PAO1 and PA14. These findings form a basis for defining defined co-cultures that can self-mEDIATE electron transfer.
In chapter four, the role of the electrochemical potential on the production of the phenazine redox mediators was determined. In order to effectively produce and use this redox mediators, it is important to understand how the terminal electron acceptor influences their production and usage. The results obtained here revealed that the applied potential influences the levels of phenazine production and the electroactivity. Not only the redox mediator production is influenced but also the overall electron transfer strategy of \textit{P. aeruginosa} is influenced. These findings provide pertinent insight into appropriately poising the electrode to harness the potential of phenazines as redox mediators. This report will stimulate more investigations into the influence of the potential applied on the central physiology of \textit{P. aeruginosa}. Answering the following questions will provide more insight for biotechnological application:

i) How does the central carbon metabolism change with regard to the applied potential?

ii) Are there differences in the potential-related physiology with different substrates?

iii) Can we steer the physiology of \textit{P. aeruginosa} by for instance applying different potentials to achieve different physiological states in one experiment?

Chapter five was an analysis of the overall carbon source influence on virulence factors production. The production of virulence factors with glucose and 2,3-BD (the standard substrates used in chapter 3) was compared with succinate and octanoate without the influence of the electrode. The signalling \textit{via} QS that leads to virulence factors production was also assessed through the quantification of the AHL signal molecules. It was confirmed that 2,3-BD stimulated more phenazine production compared to glucose. Using a rich medium mimicking the cystic fibrosis lung, it was confirmed \textit{(in vitro)} that 2,3-BD influences virulence factors production in a host environment. It was also observed that octanoate stimulated production of very high amounts of PYO under aerobic conditions. A positive correlation of the amount of signal
molecules to the levels of virulence factors production was not entirely observed. It seems, therefore, that nutritional influence on virulence generation is a concerted signalling of QS together with other regulation systems. In addition to other reports on nutritional influence on virulence factors production, the findings in this chapter provide valuable information that can be considered during the development of remedies. These findings will also be important in designing syntrophic and synergistic networks for application in substrate oxidation for electricity generation; for instance when using the complex wastewater substrates. To comprehensively understand and apply the nutrition-based virulence generation and harness its potential in remedies development and BES, the following questions need to be addressed next:

i) Which additional regulatory mechanisms act concertedly with QS or regulate it during the nutritional detection and signalling and subsequently virulence factors production?

ii) How are the nutritional cues perceived, and what does the perception of a certain carbon source mean to the cell?

In chapter six, the main endeavour was to determine whether, 2,3-BD is indeed a signal that interacts with the signalling systems that control virulence factors production. Since \textit{P. aeruginosa} produces some amounts of acetoin, diacetyl and 2,3-BD, it was imperative to find out whether the endogenously produced 2,3-BD also stimulates virulence factors production. Mutants lacking the butanoate pathway (2,3-BD and related metabolites synthesis pathway) and the 2,3-BD catabolism pathway were generated. Attempts to characterise the mutants in the standard AB medium were however futile. The mutants lacking the butanoate pathway were impaired in the metabolism of glucose. Further characterisation under more controlled conditions will be required to comprehensively address this question.
Chapter seven focused on identifying the two-component system(s), which 2,3-BD interacts with to propagate it signalling. Knowledge on 2,3-BD signalling in *Arabidopsis* *via* two-component systems was borrowed. In *Arabidopsis*, 2,3-BD interacts with two-component systems coded for by the genes *ein2* and *cre1*. Protein sequences of these systems were compared with those of *P. aeruginosa*. The two-component systems RcsC/RcsB and PvrS/PvrR were found to be similar to CRE1. Knock-out mutants of these systems showed reduced production of phenazines with 2,3-BD. These systems seem to regulate phenazine production differently depending on the carbon source. This first report concerning the mechanism of 2,3-BD signalling will go a long way towards actively steering synergistic interactions. It will also elicit further investigations into understanding signalling that forms the background of nutritional influence on virulence factors production. For these finding to be biotechnologically viable a number of questions remain to be answered:

i) Which of these two systems is responsible for down regulation and up regulation?

ii) How does the signalling from RcsC/RcsB and PvrS/PvrR proceed to activate the genes for phenazine production?

iii) Do all carbon sources, which stimulate virulence factors production, use RcsC/RcsB and PvrS/PvrR, or do they all employ specific two-component systems?
9 Appendices

9.1 Appendix 1: Phenazine production time profiles for Chapter 5-section 5.3.1.3.

Figure 9.1: Phenazine production over timer in medium containing glucose, 2,3-BD and a mixture of glucose/2,3-BD (Chapter 4-section 5.3.1.3).
Figure 9.2: Phenazine production over time by cultures provided with (top) succinate and (bottom) octanoate (Chapter 5-section 5.3.1.3).
### Primers used in the sequencing of phenazine operons (Chapter 3).

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<th>Primer name</th>
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<th>Application</th>
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<td>OP1-1 (for)</td>
<td>TGCCCGGTGGTTGCTAGCG</td>
<td>KRP1: phz operon 1 and 2 PCR amplification</td>
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<td>OP1-2 (rev)</td>
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<td>OP2-1 (for)</td>
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<td>GGAAGTAGCGCGTCGTTC</td>
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<td>TGGAAACCGTCGTAATGG</td>
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<td>OP1-8916-S11</td>
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<td>OP2-1-S1</td>
<td>TCGTCTGGCGCGGAATTCTG</td>
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<td>OP2-940-S2</td>
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<td>OP2-1701-S3</td>
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<td>OP2-2624-S4</td>
<td>ATCGGAGTACCGACAGATG</td>
<td>KRP1: phz Operon 2 sequencing</td>
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<td>OP2-3518-S5</td>
<td>GATCTGCGCGTAGGGAATC</td>
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<td>OP2-7244-S9</td>
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Table 9.2: Primers used in the generation and confirmation of the butanoate and 2,3-BD oxidation pathway mutants (Chapter 7)

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<tr>
<th>Primer name</th>
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<tr>
<td>als-Left-for</td>
<td>gaatcggagctcggtacCTACCAGAATGGCGAATTG</td>
<td>Generation and confirmation of PA14 <em>als</em> mutant</td>
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<tr>
<td>als-Left-rev</td>
<td>atgcacccTGGCCATCTGCGTTCTAC</td>
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<tr>
<td>als-Right-for</td>
<td>agatggccaGGGTGACTAGTGCAGATGGCGAATTG</td>
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</tr>
<tr>
<td>als-Right-rev</td>
<td>gtcgactttagagatccCGCCTACCAACTCGACTAC</td>
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<tr>
<td>ilv1-Left-for</td>
<td>gaatcggagctcggtacAGCCGATGATGGCAACTTTTC</td>
<td>Generation and confirmation of a PA14 <em>ilv1</em> mutant</td>
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<tr>
<td>ilv1-Left-rev</td>
<td>acgcgctctGGTTCCCGCATGAAGAACGCTG</td>
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<tr>
<td>ilv1-Right-for</td>
<td>gcgaagacgACAGCGGCGATGATGATATGC</td>
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<tr>
<td>ilv1-Right-rev</td>
<td>gtcgactttagagatccGTCCTGGGAATGCGCTAAGTTG</td>
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<tr>
<td>ilv1-Gen-L-rev</td>
<td>CGTCCGATGTCCTTTGATGTTG</td>
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<tr>
<td>ilv-KO-rev</td>
<td>TGGGCATACGCTCAACTTC</td>
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<tr>
<td>ilvG-Left-for</td>
<td>GTAATCTGAATTCGAGCTTGCCGCCCTCTCTCGTAG</td>
<td>Generation and confirmation of the PA14 <em>ilvG</em> mutant</td>
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<td>ilvG-Left-rev</td>
<td>CGATCCGATATGCACTAGACGCGAATCG</td>
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<tr>
<td>ilvG-Right-for</td>
<td>CTACTGACATACGGATCGGAGATACCCCTCGCGCAAGGC</td>
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<td>ilvG-Right-rev</td>
<td>CCCGGGTACCAGGAGCTTCGAAATGGCCGGGGAGAAG</td>
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<td>ilvG-Gen-L-rev</td>
<td>TCGGCCCCACTACCTACTCTCC</td>
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<td>ilvG-pEMG-rev</td>
<td>CAGCGCATCCGCTCTCTATCG</td>
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<td>ilvG-pEMG-for</td>
<td>CCTGCGATCCCGACGAGATTTG</td>
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<td>ilv-G-Right-rev</td>
<td>AGGAGGTGACGCTGGAGTTC</td>
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<td>ilv-G-KO-rev</td>
<td>GCGCCTACGCTTTCTTG</td>
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<tr>
<td>aco-Left-for</td>
<td>ATCTGAATTCGAGCTTGTCCTCAGAACGCGGCTGCACTC</td>
<td>Generation and confirmation of the 2,3-BD catabolism pathway</td>
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<tr>
<td>aco-Left-rev</td>
<td>CTTAGGCTCTCTAGTAGGAGAACAACAGAG</td>
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<td>aco-Right-for</td>
<td>TTCACCAACTAGGAGGCTCTAAGGAGGAAAC</td>
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<td>CCCGGGTACCAGGAGCTTCGACACCACCAGGTAGTG</td>
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<td>aco-KO-rev</td>
<td>GTGATCGCCCTGAGAGTGC</td>
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Table 9.3: Primers for the generation of the two-component system mutants in the PAPI Island

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<tr>
<th>Primer name</th>
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<tr>
<td>TPAPI-left-for</td>
<td>GGGTAATCTGAATTCCAGCTTGCTCGAGGCAA TAATCGC</td>
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<td>TPAPI-Left-rev</td>
<td>TCTTACAGCCAGCGACCGACCGCATTGATG</td>
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<td>TPAPI-Right-for</td>
<td>CCTGGTCCGTGCTCTGTAAGAAATTTCTTCA AC</td>
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<tr>
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<td>CCCGGGTACGAGCTAGTCATGGTCTGGTCA TCCAG</td>
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<tr>
<td>TPAPI-Gen-L-for</td>
<td>GACGGCAGTGCGATGAACC</td>
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<tr>
<td>TPAPI-pEMG-rev</td>
<td>CTGGAAAGCGGGCAGTCATGGTCTGGTCA TCCAG</td>
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<tr>
<td>TPAPI-pEMG-for</td>
<td>CTGGATGCGGGACGGATTTG</td>
</tr>
<tr>
<td>TPAPI-Gen-Right-rev</td>
<td>CGCGGCACTACGAATAAC</td>
</tr>
<tr>
<td>TPAPI-KO-rev</td>
<td>AAGATAGTACTGGCCGATGAC</td>
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</table>
9.3 Appendix 3: Motility assay (Chapter 5-section 5.3.1.4.1)

![Swimming, Swarming, Twitching with Glucose and 2,3-BD for strain PA14](image)

Figure 9.3: Swimming, swarming and twitching with glucose and 2,3-BD for strain PA14. For swimming, the samples from the conditions of growth were inoculated separately since they need more space during the swimming. For the distances covered see Table 5.3 in chapter 5-section 5.3.1.4.1.

![Swimming, Swarming, Twitching with Equimolar Glucose/2,3-BD for strain PA14](image)

Figure 9.4: Swimming, swarming and twitching with equimolar glucose/2,3-BD for strain PA14. For the distances covered see Table 5.3 in chapter 5-section 5.3.1.4.1.
Figure 9.5: Swimming, swarming and twitching succinate and octanoate for strain PA14.
Figure 9.6: Swimming, swarming and twitching with glucose and 2,3-BD for strain KRP1. For swimming, the samples from the conditions of growth were inoculated separately since they need more space during the swimming. For the distances covered see Table 5.3 in chapter 5-section 5.3.1.4.1

Figure 9.7: Swimming, and twitching with equimolar glucose/2,3-BD for strain KRP1. For the distances covered see Table 5.3 in chapter 5-section 5.3.1.4.1
Figure 9.8: Swimming, swarming and twitching succinate and octanoate for strain KRP1. For the distances covered see Table 5.3 in chapter 5-section 5.3.1.4.1
9.4 Appendix 4: PCA-like compound produced by RcsC/RcsB and PvrS/PvrR mutant with glucose (Chapter 6-section-7.2.1.1)

Figure 9.9: a) Chromatogram showing PCA and the PCA-like molecule at 17.5 and 19.0 minutes respectively and b) the spectrum of the unknown compound.
References


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Mobile: 015211237986
Email: bosireerick@yahoo.com

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<td>August 2007-December 2010</td>
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Workshops and training
May 2011 | Animal movement and migration, National Museums of Kenya and USGS

Work experience
April 2016-October 2016 | Research assistant, Institute of Applied Microbiology, RWTH University Aachen, Germany. Conducting research,
August 2011-May 2012  **Assistant lecture**, Kenya Methodist University Meru. Teaching microbiology

May 2011-May 2012  **Part time Lecturer** in Zoology Department, Kenyatta University, Nairobi, Kenya. Teaching Laboratory methods and techniques in Zoology and cell biology.

August 2009-May 2012  **Part time lecturer** in the Department of Education and Natural Science, Mt Kenya University, Thika, Kenya. Teaching, Plant taxonomy and Human anatomy.

January 2011-May 2012  **Adjunct lecturer** at the University of Eastern Africa Baraton, Eldoret, Kenya-Nairobi extension and Nairobi hospital

**Languages**

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**Awards and scholarships**

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<tr>
<td>March 2016</td>
<td>Deutscher Akademischer Austausch Dienst (DAAD) Travel Grant Award for the 2016 ASM Microbes meeting in Boston, USA.</td>
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<tr>
<td>May 2015</td>
<td>ASM students and postdocs travel grant (not used).</td>
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<tr>
<td>September 2014</td>
<td>Best oral communication award by the European conference of the International Society for Microbial Electrochemistry and Technology (EU-ISMET). Awarded during the 2nd EU-ISMET meeting 3-5 September 2014 at the University of Alcalà Spain.</td>
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<tr>
<td>June 2012 – March 2016</td>
<td>Deustcher Akademischer Austausch Dienst (DAAD) scholarship for Doctoral research and training.</td>
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**Skills**

**Scientific skills**

- **Microbiology skills**: Cell culture, working with biosafety II organisms, small scale BES-reactor fermentation.
- **Molecular biology skills**: PCR, primer design, cloning using Gibson assembly, mutant generation via homologous recombination.
- **Bio-electrochemistry skills**: reactor setup for electrochemical fermentation, cyclic voltammetry, chronoamperometry.
- **Analytical skills**: HPLC, GC-MS, spectrometry.
- **Immunology**: ELISA, FACS.

**Other skills**

- Graph pad, Origin, Excel, MS-Word, Power Point, Clone Manager.
### Memberships

i) Kenya Society of Immunology (KSI) (since 2009)
ii) Ecological Society of East Africa (ESEA) (since 2011)
iii) International Society for Microbial Electrochemistry and Technology (ISMET) (since 2014)
iv) American Society for Microbiology (ASM) (since 2015)

### Publication List

#### Journal Publications

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<tr>
<td>Bosire EM., Blank LM. and Rosenbaum MA.</td>
<td>Synergistic Virulence Generation by <em>Pseudomonas aeruginosa</em> Strains, Implications for Application in Bioelectrochemical Systems.</td>
<td>ASM microbe 2016, 16th-20th June 2016, Boston, USA. Poster presentation</td>
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<tr>
<td>Bosire EM. and Rosenbaum M.</td>
<td>Exploiting the potential of interspecies communication and synergism in <em>Pseudomonas aeruginosa</em> co-cultures for electricity generation.</td>
<td>9th-11th March 2014. Marburg, Germany. Poster presentation</td>
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<td>Bosire E. and Rosenbaum M.</td>
<td>Redox mediator electron transfer using <em>Pseudomonas aeruginosa</em> based co-cultures.</td>
<td>DBU German workshop on Microbial Electrochemistry, 19th-</td>
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175
20th November 2013. Osnabrück, Germany. Oral presentation

**Accepted abstracts**


ASM microbe 2017, 1st-5th June 2017, New Orleans, USA.

Oral presentation.

---

**REFERENCES**

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5/25/2017

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