

**Endogenous production and detoxification
of a potent cytotoxin, nitric oxide, in
Salmonella enterica serovar Typhimurium
and *Escherichia coli***

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with the requirements of the degree of Doctor of Philosophy

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Abstract

Salmonella serovars are harmful enteric pathogens of economical and clinical importance that possess sophisticated strategies to rapidly adapt to various host (human and animal) and non-host (soil, water and industrial) environments. Nitrosative stress, in the form of RNS such as the potent cytotoxin NO, is an important stress in the *Salmonella* lifecycle. *Salmonella* is exposed to exogenous NO, produced by activated macrophages as part of the host immune response and to endogenous NO, produced during anaerobic nitrate respiration. *Salmonella* employs three known enzymes (HmpA, NrfA and NorVW) to detoxify NO to less toxic compounds, including the neuropharmacological agent and greenhouse gas N₂O. The production of endogenous NO and N₂O have been predominantly studied in denitrifying soil bacteria and have been widely neglected in enteric bacteria.

Here, the physiological and molecular mechanisms involved in endogenous NO production and detoxification were examined in the pathogenic *Salmonella enterica* serovar Typhimurium and laboratory *Escherichia coli* (*E. coli*) strains. Significant differences in N₂O production were observed between the two genera and between the tested *E. coli* strains, although they possess identical nitrate respiration systems. The reason for this was found to be transcriptional, with *narG* expression having the major impact. In addition, our results indicate that a weak nitrous oxide reductase exists in *Salmonella*; a process that was believed to be restricted to certain soil bacteria, archaea and fungi that possess the enzyme NosZ. Furthermore, the contribution of selected NsrR regulon genes, to endogenous N₂O production of *Salmonella* was determined and revealed that HmpA and the Hcp-Hcr operon are both crucial for high N₂O levels. These findings provide new insights into host-pathogen interactions, which could potentially lead to new treatment strategies for *Salmonella* infections, help to increase food safety and provide new mitigation strategies to reduce global warming.

Dedicated to my Mum and Dad

Table of contents

Abstract	2
List of tables	9
List of figures	10
List of abbreviations	13
Acknowledgements	15
1 Introduction	16
1.1 The biogeochemical nitrogen cycle	17
1.2 Enterobacteriaceae	21
1.2.1 Nomenclature	21
1.2.2 Characteristics of Enterobacteriaceae	24
1.2.3 <i>Escherichia coli</i>	25
1.2.4 <i>Salmonella</i>	26
1.3 Enteric bacteria and anaerobic metabolism.....	28
1.3.1 Life in the mammalian gastrointestinal tract.....	29
1.4 Nitrate respiration in <i>S. Typhimurium</i> and <i>E. coli</i>	30
1.4.1 Overview of nitrosative stress	30
1.4.2 NO characteristics and reactivity	31
1.4.3 NO generation in the host environment	33
1.4.4 Endogenous NO production during denitrification processes	37
1.4.5 NO protection and detoxification.....	44
1.5 Thesis overview.....	58
1.5.1 Research gap	58
1.5.2 Aims.....	58
2 Materials & Methods	59
2.1 Materials.....	60
2.2 Bacterial strains.....	60
2.2 Bacterial culture conditions.....	61
2.2.1 Media	61
2.2.2 Overnight culture	61
2.2.3 Freezer stocks	61

2.3 Polymerase chain reaction (PCR)	62
2.3.1 Colony PCR	62
2.3.2 PCR product purification	62
2.4 Plasmid purification (Miniprep)	63
2.5 Green plates	64
2.6 Ammonia testing	64
2.7 Anaerobic batch culture	65
2.7.1 Hungate batch procedure	66
2.8 Continuous culture	67
2.8.1 Chemostat procedure	69
2.9 Spectrophotometry	70
2.10 Agarose gel electrophoresis	70
2.11 GC	71
2.11.1 Sampling and storage of N ₂ O	71
2.11.2 N ₂ O measurement	71
2.11.3 Conversion of N ₂ O ppm (by mass) to μM	72
2.11.4 Henry's law constant	74
2.12 N ₂ O electrode	76
2.12.1 Electrode preparation	76
2.12.2 Cell preparation	80
2.12.3 Electrode procedure	80
2.12.4 Preparation of an aqueous saturated N ₂ O solution	81
2.13 Drymass constant	82
2.14 HPLC	83
2.15 Knock-out mutant construct via the λ-red system	83
2.15.1 Generation of FRT-flanked resistance gene construct	86
2.15.2 Electroporation	86
2.15.3 Knock-out mutant verification	88
2.15.4 P22 transduction	89
2.15.5 Removal of an antibiotic resistance cassette	90
2.16 RNA extraction and qRT-PCR	90
2.16.1 Cell harvesting	91
2.16.2 RNA extraction and purification	91

2.16.3 Assessment of RNA quality and quantity	92
2.16.4 qRT-PCR primer design.....	92
2.16.5 Genomic DNA preparation.....	93
2.16.6 qRT-PCR	94
2.17 Microarray	95
2.17.1 Direct labelling of sample RNA	96
2.17.2 Direct labelling of reference DNA.....	97
2.17.3 Hybridisation	98
2.17.4 Scanning and analysis.....	99
3 Differences in <i>narG</i> expression levels contribute to variations in nitrous oxide emissions between <i>Salmonella</i> Typhimurium and laboratory strains of <i>Escherichia coli</i>	100
3.1 Introduction.....	101
3.2 Aim	105
3.3 Experimental design	105
3.3.1 Anaerobic Hungate batch	105
3.3.2 Drymass constant	105
3.3.3 Continuous chemostat cultures.....	106
3.5 Results	106
3.5.1 N ₂ O production of nitrate-sufficient batch cultures	106
3.5.2 Nitrate respiration of nitrate-sufficient continuous cultures.....	109
3.5.3 Nitrate respiration of nitrate-limited continuous cultures	117
3.5.4 The importance of NarG during anaerobic nitrate respiration.	123
3.6 Discussion	129
3.6.1 Anaerobic nitrate respiration of Hungate batch cultures	129
3.6.2 Anaerobic nitrate respiration of chemostat cultures	130
3.6.3 The importance of NarG	133
3.7 Future work	134
4 Investigating the existence of a nitrous oxide reductase in <i>Escherichia coli</i> and <i>Salmonella</i>	135
4.1 Introduction.....	136
4.2 Aim	139
4.3 Experimental design.....	139

4.3.1 Anaerobic Hungate batch supplemented with acetylene	139
4.3.2 Anaerobic Hungate batch at different pH levels	140
4.3.3 Anaerobic Hungate batch of $\Delta cueO$ cultures	140
4.3.4 N ₂ O electrode	140
4.3.5 Mutant constructs.....	141
4.4 Results	143
4.4.1 The effect of acetylene on N ₂ O production of nitrate-sufficient batch cultures	143
4.4.2 The effect of pH on N ₂ O production of nitrate-sufficient batch cultures.....	146
4.4.3 N ₂ O production of nitrate-sufficient batch cultures of <i>Salmonella</i> and <i>E. coli</i> $\Delta cueO$ mutants	148
4.4.4 N ₂ O consumption in <i>Salmonella</i>	152
4.5 Discussion	155
4.5.1 N ₂ OR inhibition experiments	155
4.5.2 Search for the N ₂ O reductase enzyme	157
4.6 Future work	158
5 The NsrR regulon of <i>Salmonella</i> SL1344 $\Delta nsrR$ during anaerobic nitrate respiration	159
5.1 Introduction.....	160
5.2 Aim	161
5.3 Experimental design	163
5.3.1 Continuous chemostat cultures.....	163
5.3.2 Microarray	163
5.4 Results	164
5.4.1 Nitrate-sufficient chemostat cultures of SL1344 WT vs. $\Delta nsrR$	164
5.4.2 Nitrate-limited chemostat cultures of SL1344 WT vs. $\Delta nsrR$	169
5.4.3 Microarray analysis of SL1344 WT vs. $\Delta nsrR$	174
5.5 Discussion	196
5.6 Future work	199
6 The contribution of the NsrR regulon to endogenous N₂O production in <i>Salmonella</i> Typhimurium	200
6.1 Introduction.....	201

6.2 Aim	201
6.3 Experimental design	201
6.3.1 Anaerobic Hungate batch of NsrR regulon members	201
6.4 Results	202
6.4.1 The contribution of the <i>Salmonella</i> NsrR regulon to N ₂ O production.....	202
6.4.2 N ₂ O production of nitrate-sufficient batch cultures of <i>Salmonella nar</i> , <i>nap</i> and <i>hcp</i> mutants	208
6.5 Discussion	210
6.6 Future work	211
7 General discussion.....	213
7.1 Context.....	214
7.2 <i>Salmonella</i> and the host microbiota	215
7.3 <i>Salmonella</i> and the non-host environment.....	218
7.4 Concluding remarks	220
8 References.....	222
9 Appendix.....	248
A) Media composition and supplements.....	249
B) Hybridisation for microarrays	251
C) N ₂ O electrode data (representative graphs)	252
D) Growth in anaerobic Hungate tubes under nitrate-sufficient conditions ..	258
E) Microarray table	259
F) Growth curves NsrR regulon.....	262

List of tables

Table 1 Wild-type strains used in this work	60
Table 2 PCR reaction components.....	63
Table 3 PCR programme details	63
Table 4 List of primers used for the construction of knockout mutants	86
Table 5 List of verification primers for knockout mutant.....	88
Table 6 Real time quantitative PCR primers.....	93
Table 7 qRT-PCR programme details	94
Table 8 Preparation of hybridisation buffer for 8 x array microarray slide.....	98
Table 9 Modified from Cole (2012). Selected genes and their respective regulons in <i>E. coli</i> and <i>S. Typhimurium</i>	104
Table 10 Nitrate, nitrite and N ₂ O steady state rates of chemostat cultures.....	116
Table 11 Schematic representation of qRT-PCR data of <i>S. Typhimurium</i> and <i>E. coli narG</i> and <i>napA</i> expression levels during anaerobic nitrate respiration.....	122
Table 12 N ₂ O reduction rates (qcN ₂ O) using a modified Clark electrode.	154
Table 13 NsrR repressed genes with limited knowledge about their function in nitrosative stress defence.	162
Table 14 Genes more than 2-fold higher/lower transcribed in <i>S. Typhimurium</i> WT anaerobically vs. aerobic growth conditions from G ⁺ /N ⁻ chemostat cultures.	180
Table 15 Genes more than 2-fold higher/lower transcribed in <i>S. Typhimurium</i> Δ <i>nsrR</i> vs. WT aerobically under G ⁺ /N ⁻ conditions.	188
Table 16 Genes more than 2-fold higher and lower transcribed in <i>S. Typhimurium</i> Δ <i>nsrR</i> vs. WT anaerobically under G ⁺ /N ⁻ conditions.	190
Table 17 List of genes that were more than 2-fold higher transcribed in <i>S. Typhimurium</i> Δ <i>nsrR</i> vs. WT aerobically as well as anaerobically under G ⁺ /N ⁻ conditions.....	193

List of figures

Figure 1 The biogeochemical nitrogen cycle.....	18
Figure 2 Modified from Groisman and Ochman (1997). Phylogenetic relationships among enteric bacteria	23
Figure 3 Adapted from Runkel <i>et al.</i> (2013). Schematic illustration of the nitrate respiration and NO detoxification pathways of <i>Salmonella</i> and <i>E. coli</i>	38
Figure 4 Adapted from Arkenberg <i>et al.</i> (2011). Truncated denitrification pathways in <i>Escherichia coli</i> and <i>Salmonella</i> Typhimurium.....	39
Figure 5 Schematic batch culture growth.	66
Figure 6 Schematic chemostat (A) and New Brunswick Scientific Bioflow 300 chemostat, used in this study (B).	68
Figure 7 Electrode disc preparation.	78
Figure 8 Set up of N ₂ O electrode.	79
Figure 9 Modified from (Datsenko and Wanner, 2000). Knock-out mutation via the λ -red system	85
Figure 10 Schematic microarray slide (A) and procedure (B).	96
Figure 11 Growth and N ₂ O production of anaerobic batch cultures during nitrate respiration.	108
Figure 12 Drymass constant.	110
Figure 13 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-limited/nitrate-sufficient continuous culture of <i>E. coli</i> MC1000.	112
Figure 14 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-limited/nitrate-sufficient continuous culture of <i>E. coli</i> MG1655.	113
Figure 15 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-limited/nitrate-sufficient continuous culture of <i>E. coli</i> W3110.	114
Figure 16 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-sufficient/nitrate-limited continuous culture of <i>E. coli</i> MC1000.	119
Figure 17 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-sufficient/nitrate-limited continuous culture of <i>E. coli</i> MG1655.	120
Figure 18 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-sufficient/nitrate-limited continuous culture of <i>E. coli</i> W3110.	121

Figure 19 Growth and N ₂ O production of anaerobic batch cultures of <i>S. Typhimurium</i> wild-type and a <i>narG</i> mutant during nitrate respiration.....	125
Figure 20 Competition chemostat of <i>S. Typhimurium</i> WT vs. <i>ΔnarG</i>	126
Figure 21 qRT-PCR analysis of anaerobic batch cultures of <i>S. Typhimurium</i> wild-type and a <i>narG</i> mutant during nitrate respiration.	128
Figure 22 Agarose gel electrophoresis images of the PCR verification of <i>cueO</i> deletion mutants.....	142
Figure 23 The effect of acetylene on growth and N ₂ O production of anaerobic batch cultures of <i>Paracoccus</i> during nitrate respiration.	144
Figure 24 The effect of acetylene on growth and N ₂ O production of anaerobic batch cultures of <i>Salmonella</i> and <i>E. coli</i> during nitrate respiration.....	145
Figure 25 The effect of pH on growth and N ₂ O production of anaerobic batch cultures during nitrate respiration.....	147
Figure 26 Growth and N ₂ O production of anaerobic batch cultures of <i>E. coli</i> WT vs. <i>ΔcueO</i> during nitrate respiration.....	150
Figure 27 Growth and N ₂ O production of anaerobic batch cultures of <i>S. Typhimurium</i> WT vs. <i>ΔcueO</i> during nitrate respiration.....	151
Figure 28 N ₂ O reduction in <i>Salmonella</i> SL1344.....	153
Figure 29 Nitrate consumption and nitrite and nitrous oxide production in a nitrate-sufficient/glycerol-limited continuous culture of <i>S. Typhimurium</i> SL1344.	166
Figure 30 Nitrate consumption and nitrite and nitrous oxide production in a nitrate-sufficient/glycerol-limited continuous culture of <i>S. Typhimurium</i> SL1344 <i>ΔNsrR</i>	167
Figure 31 qRT-PCR of nitrate-sufficient/glycerol-limited continuous culture of <i>S. Typhimurium</i> SL1344 WT and <i>ΔnsrR</i>	168
Figure 32 Nitrate consumption and nitrite and nitrous oxide production in a glycerol-sufficient/nitrate-limited continuous culture of <i>S. Typhimurium</i> SL1344.	170
Figure 33 Nitrate consumption and nitrite and nitrous oxide production in a glycerol-sufficient/nitrate-limited continuous culture of <i>S. Typhimurium</i> SL1344 <i>ΔnsrR</i>	171
Figure 34 qRT-PCR of glycerol-sufficient/nitrate-limited continuous culture of <i>S. Typhimurium</i> SL1344 WT and <i>ΔnsrR</i>	173
Figure 35 Relative percentage of genes up-/down-regulated in <i>S. Typhimurium</i> WT during anaerobiosis compared to aerobic growth.....	176
Figure 36 Relative percentage of genes up-/down-regulated in <i>S. Typhimurium</i> <i>ΔnsrR</i> during anaerobiosis compared to aerobic growth.	177

Figure 37 Relative percentage of genes that were higher/lower transcribed in <i>S. Typhimurium</i> $\Delta nsrR$ vs. WT during aerobic growth.....	185
Figure 38 Relative percentage of genes that were higher/lower transcribed in <i>S. Typhimurium</i> $\Delta nsrR$ vs. WT during anaerobic growth.....	186
Figure 39 Heat map representing the different expression levels of selected genes of <i>S. Typhimurium</i> WT and <i>nsrR</i> mutant chemostat cultures during aerobic and anaerobic growth.....	193
Figure 40 Nitrous oxide production of nitrate-sufficient anaerobic batch cultures of selected <i>Salmonella</i> NsrR regulon members.....	203
Figure 41 Nitrous oxide production of nitrate-limited anaerobic batch cultures of selected <i>Salmonella</i> NsrR regulon members.....	204
Figure 42 Nitrous oxide production of anaerobic batch cultures of selected <i>Salmonella</i> $\Delta nsrR$ mutants.....	206
Figure 43 Nitrous oxide production of nitrate-sufficient anaerobic batch cultures of <i>Salmonella nar</i> , <i>nap</i> and <i>hcp</i> mutants.....	209
Figure 44 Modified from Rowley <i>et al.</i> (2012). Schemes for anaerobic nitrate respiration in <i>Salmonella Typhimurium</i> under different growth conditions.....	221

List of abbreviations

(v/v)	Volume per volume
[Fe-S]	Iron sulphur cluster
Amp	Ampicillin
bp	Base pair
C ₂ H ₂	Acetylene
CDC	Centre for Disease Control and Prevention
cDNA	Complementary DNA
cfu	Colony forming unit
cGMP	Cyclic guanosine monophosphate
Cm	Chloramphenicol
C _t value	Threshold cycle value
dH ₂ O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
EtOH	Ethanol
FNR	Fumarate and nitrate reductase regulator
Fur	Ferric uptake regulator
G ⁺ /N ⁻	Glycerol-sufficient/ nitrate -limited
GC	Gas chromatography
GIT	Gastrointestinal tract
GNSO	S-nitrosogluthatione
H	Hour(s)
H ₂ O ₂	Hydrogen peroxide
Hcp	Hybrid cluster protein
Hcy	Homocysteine
HGT	Horizontal gene transfer
HNO ₂	Nitrous acid
HPLC	High performance liquid chromatography
IFN-γ	Gamma interferon
IL-1	Interleukine-1
iNOS	Inducible nitric oxide synthase
IPCC	The Intergovernmental Panel on Climate Change
Kb	Kilobase-pair
KEGG	Kyoto Encyclopaedia of Genes and Genomes
Km	Kanamycin
LB	Luria-Bertani
LPS	Lipopolysaccharides
MCO	Multicopper oxidase
MetR	Methionine Regulator
N ⁺ /G ⁻	Nitrate-sufficient/glycerol-limited
N ₂	Dinitrogen
N ₂ O	Nitrous oxide
N ₂ OR	Nitrous oxide reductase

NaOH	Sodium hydroxide
NFκB	Nuclear factor-kappaB
NH ₄ ⁺	Ammonium
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO ₂	Nitrogen dioxide radical
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate
NorR	Nitric oxide reductase transcription regulator
NsrR	Nitric oxide-sensitive repressor
O ₂	Oxygen
O ₂ ⁻	Superoxide anion
OD	Optical density
OH•	Hydroxyl radical
ONOO ⁻	Peroxynitrite
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Phox	NADPH phagocyte oxidase
PPM	Parts per million
qcNO ₂ ⁻	Rate of nitrite accumulation
qcNO ₃ ⁻	Rate of nitrate consumption
qpN ₂ O	Rate of nitrous oxide production
qRT-PCR	Quantitative real-time polymerase chain reaction
REL	Relative expression level
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
<i>S. Typhimurium</i>	<i>Salmonella enterica</i> serovar Typhimurium
SCV	<i>Salmonella</i> containing vacuole
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SPI	<i>Salmonella</i> pathogenicity island
sRNA	Small RNA
st. dev.	Standard deviation
T3SS	Type III secretion system
TBE	Tris-buffered EDTA
TCA	Tricarboxylic acid cycle
TLR	Toll-like Receptor
TNF-α	Tumor necrosis factor alpha
Tris	Tris(hydroxymethyl)-aminomethane
UTI	Urinary tract infections
WHO	World Health Organization
WT	Wild-type

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1 Introduction

1.1 The biogeochemical nitrogen cycle

The nitrogen cycle is one of the most important nutrient cycles in our environment. Nitrogen is required by all organisms to synthesize nucleic acids, amino acids, proteins and other essential cofactors. Man-made alterations, such as burning fossil fuels and the increased use of fertilizer for agricultural purposes, have increased awareness about the potential harm for the environment and thus research interest in this area has increased in recent times (Moenne-Loccoz and Fee, 2010). One concern is the production of nitrous oxide (N_2O) by terrestrial and marine microbes; a greenhouse gas with 300 times higher global warming potency than CO_2 and an atmospheric lifetime of approximately 150 years (Richardson et al., 2009). The Intergovernmental Panel on Climate Change (IPCC) estimated a 20% increase of atmospheric N_2O over the past century with an annual increase at a rate of 0.2-0.3% (Thomson et al., 2012).

Figure 1 shows the nitrogen cycle and its diverse redox reactions, which are mainly accomplished by bacteria. Nitrogen is present in all of its oxidation states; from the most strongly reduced state -3 in the form of ammonia, to the highest oxidation state of +5 in the form of nitrate ions. Many of the enzymes that drive these reactions contain metal ions in their active centres (Richardson and Watmough, 1999, Zumft, 1997).

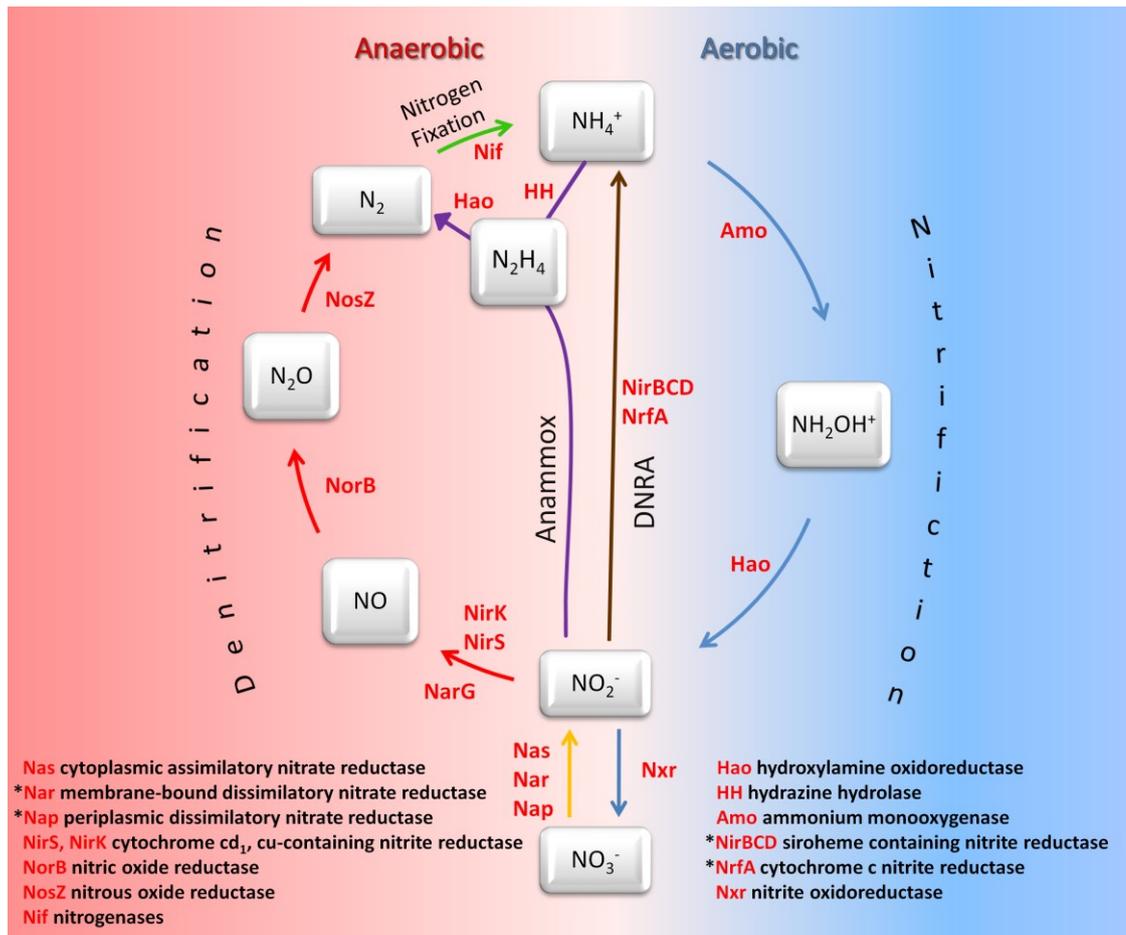


Figure 1 The biogeochemical nitrogen cycle.

The biogeochemical nitrogen cycle depends on several redox reactions that are driven by the respective enzymes. Enzymes that conduct these conversions include various nitrate reductases (Nas, NarG, NapA), nitrite reductases (NirBD, NrfA), nitric oxide reductase (NorB), the nitrous oxide reductase (NosZ), the nitrogenase (Nif), the ammonium monooxygenase (Amo), the hydroxylamine oxidoreductase (Hao), the nitrite oxidoreductase (Nxr) and the hydrazine hydrolase (HH). Enzymes marked with an asterisk are present in *E. coli* and *Salmonella*. The NO detoxification pathways, alongside their enzymes are left out here for illustrative reasons.

Atmospheric nitrogen, also known as dinitrogen (N_2), is highly inert due to its strong triple bond interactions between the two atoms. Thus, to be assimilated by plants and organisms, it first has to be converted into an available form such as inorganic ammonium ions (NH_4^+) or nitrate ions (NO_3^-). The process of converting dinitrogen to a chemically available form is called nitrogen fixation and is predominantly accomplished by bacteria, archaea and a few eukaryotes (e.g. legumes) that use bacteria to fix nitrogen. Under conditions of high pressure and temperature, as found near lightning bolts, N_2 can react with oxygen (O_2) to form the gaseous nitrogen oxides, nitric oxide (NO) and nitrogen dioxide (NO_2), which can eventually be converted to NO_3^- via a series of subsequent reactions with water in the rain (Canfield et al., 2010). The next step of the cycle is nitrification, the microbial oxidation of ammonium to nitrate with oxygen. At first, NH_4^+ is oxidised to hydroxylamine (NH_2OH) by organisms containing the enzyme ammonium monooxygenase (AMO). Hydroxylamine is then further oxidised to nitrite (NO_2^-) by the hydroxylamine oxidoreductase (HAO) and finally to NO_3^- by the nitrite oxidoreductase (NXR). Bacteria able to catalyse this process are called nitrifiers. Another process, not shown in Figure 1, is the conversion of NH_4^+ to N_2 with NH_2OH and N_2O as intermediates. Under anaerobic conditions nitrate is used as an alternative respiratory electron acceptor for energy generation by many microorganisms (Ye and Thomas, 2001, Stewart, 1988). Three pathways exist in which nitrate becomes converted to either NH_4^+ or N_2 . NO_2^- is the common intermediate of these processes and is produced by one of three nitrate reductases: the membrane bound - respiratory (Nar), the periplasmic - dissimilatory (Nap) or the soluble cytoplasmic - assimilatory (Nas). The pathway in which NO_3^- is reduced to NH_4^+ , via NO_2^- , is called dissimilatory nitrate reduction to ammonium (DNRA) and is driven by the nitrite reductases (NirBD) and (NrfA) (Canfield et al., 2010, Rodionov et al., 2005). The other two pathways have N_2 as their common end product. One of these pathways, anaerobic ammonium oxidation (anammox), combines NH_4^+ oxidation with NO_2^- reduction to form the intermediate hydrazine (N_2H_4), which involves the enzymes hydrazine hydrolase (HH) and NirBCD, respectively (Kartal et al., 2011). The subsequent reduction to N_2 is triggered by the enzyme HAO. The other N_2 producing pathway, denitrification, comprises the obligate intermediates NO and N_2O (Canfield et al., 2010). Enzymes involved in this

process include the nitrite reductases NirK or NirS, the nitric oxide reductase (NorB) and the nitrous oxide reductase (NosZ) (Rodionov et al., 2005). The return of N₂ to the atmosphere completes the nitrogen cycle.

A wide range of microorganisms are able to undergo denitrification, including *Paracoccus* spp., *Pseudomonas* spp., *Rhodobacter* spp., some fungi and certain archaea (Zumft, 1997). *Paracoccus denitrificans* is a member of the α -Proteobacteria and probably the best studied denitrifying soil bacteria (Felgate et al., 2012, Thomson et al., 2012). *Paracoccus* uses either the quinol-dependent Nar, located in the cytoplasm, or the periplasmic Nap to reduce NO₃⁻ to NO₂⁻. Nitrite reduction to NO proceeds with the two heme cofactor-containing *cd₁Nir* that is encoded by *nirS* (Richardson, 2008). The cytotoxic radical, NO, becomes quickly reduced to N₂O by the integral membrane nitric oxide reductase NorBC, before it gets converted to N₂ by the copper-containing NosZ in the periplasm (Pomowski et al., 2011, Field et al., 2008).

Denitrification processes are not only seen in classical denitrifiers but also in Enterobacteriaceae, such as *Salmonella* and *Escherichia coli* (*E. coli*). Similar to *Paracoccus*, *Salmonella* and *E. coli* use the Nar and Nap enzymes for the first step of anaerobic nitrate respiration. In addition, they possess the NorBC isoenzyme NorVW for NO detoxification. Besides these enzymes, the similarity in their denitrification processes ends. Unlike soil bacteria, which produce the potent greenhouse gas N₂O as an intermediate product during denitrification, enteric bacteria undergo only a truncated form of denitrification, resulting in N₂O as their end product (Figure 4) (Richardson et al., 2009, Arkenberg et al., 2011, Thomson et al., 2012, Rowley et al., 2012). N₂O emissions by denitrifying soil bacteria are well documented, while N₂O production in enteric bacteria has been widely neglected. The nitrate respiration and NO detoxification pathways of *Salmonella* and *E. coli*, which are essential for their survival in a variety of environments, will be discussed in detail in sections 1.4.4 and 1.4.5.

1.2 Enterobacteriaceae

1.2.1 Nomenclature

The Enterobacteriaceae family (also known as enteric bacteria) encompass many genera and a correspondingly larger number of species that inhabit natural (soil, marine) and industrial (sewage, dairy products) environments, as well as the gastrointestinal tract of humans and warm-blooded animals. Members of the Enterobacteriaceae family are among the most frequent clinical isolates, comprising pathogenic and commensal species (Sievert et al., 2013, Stecher et al., 2012). Some of the more familiar genera include *Escherichia*, *Salmonella*, *Shigella*, *Enterobacter*, *Klebsiella*, *Yersinia* and *Proteus*. Their phylogenetic relationships are presented in Figure 2A. Besides the well known *E. coli*, the genus *Escherichia* includes the species *Escherichia albertii*, *Escherichia blattae*, *Escherichia fergusonii*, *Escherichia hermannii* and *Escherichia vulneris* (Skerman et al., 1980, Baylis et al., 2006). *E. coli* is one of the most important model organisms in biology and medicine and by far the best characterised prokaryote. Major advancements in genetics, molecular biology, biochemistry and bacterial physiology have emerged from *E. coli* studies, especially from derivatives of the K-12 strain such as bacterial conjugation and recombination (Lederberg and Tatum, 1946). Population genetic analyses classify *E. coli* strains into four major phylogenetic groups (A, B1, B2, D) and a potential fifth group (E) that are distinct in their phenotypic characteristics, such as the ability to utilize certain sugars or their antibiotic resistance profiles (Herzer et al., 1990, Wirth et al., 2006, Gordon et al., 2008, Touchon et al., 2009). *Escherichia coli* K-12 belongs to the subgroup A and was originally isolated in 1922 from the faeces of a diphtheria patient in Palo Alto, California. The K-12 strains can be further categorised into substrains. Two common laboratory substrains, used in our work, are *Escherichia coli* K-12 subst. MG1655 and W3110. Their genomes have been published in 1997 and 2006, respectively and were identified to be almost identical (Blattner et al., 1997, Hayashi et al., 2006). Further information on the evolution of *E. coli*

phylogeny and population genetics can be found in the following review articles (Chaudhuri and Henderson, 2012, Tenailon et al., 2010).

Salmonella is a well-known medically important pathogen that is named after Dr Daniel E. Salmon, who first isolated *Salmonella choleraesuis* from a pig intestine in 1884 (Su and Chiu, 2007). However, its nomenclature has been restructured multiple times and we are now left with the *Salmonella* genera consisting of just two species, *Salmonella enterica* and *Salmonella bongori* (Grimont and Weill, 2007, Brenner et al., 2000, Reeves et al., 1989). DNA-DNA hybridisation experiments were a key development in understanding *Salmonella* taxonomy (Crosa et al., 1973). The proposed third species *Salmonella subterranean* (Shelobolina et al., 2004) was initially approved by the Judicial Commission of the International Committee of Systematic Bacteriology of the World Health Organization (WHO) in 2005, but it was shown later that this species is more closely related to *Escherichia hermannii* and does not belong to the genus *Salmonella* (Grimont and Weill, 2007). After many requests from Ezaki, Euzeby and Le Minor and Popoff, it was officially decided by the Judicial Commission in the Opinion 80 that *Salmonella enterica* should replace *Salmonella choleraesuis* as the type species of the genus *Salmonella* (Euzeby, 1999, Le Minor and Popoff, 1987, Ezaki et al., 2000, Tindall et al., 2005). *Salmonella enterica* is further divided into the six subspecies: *S. enterica* subsp. *enterica* (subspecies I), *S. enterica* subsp. *salamae* (subspecies II), *S. enterica* subsp. *arizonae* (subspecies IIIa), *S. enterica* subsp. *diarizonae* (subspecies IIIb), *S. enterica* subsp. *houtenae* (subspecies IV) and *S. enterica* subsp. *Indica* (subspecies VI) (Figure 2B).

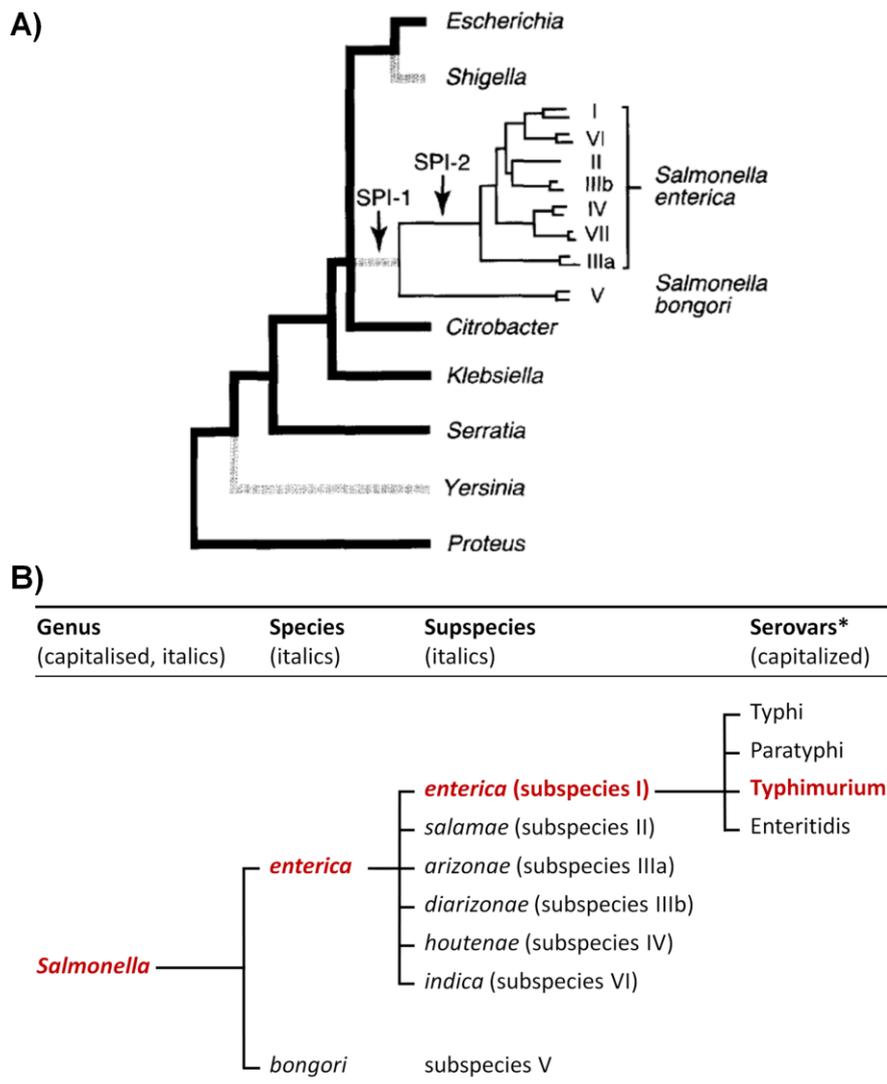


Figure 2 Modified from Groisman and Ochman (1997) Phylogenetic relationships among enteric bacteria.

A) The branches shown in grey denote taxa that are typically capable of invading eukaryotic cells. SPI-1 and SPI-2 are the *Salmonella* pathogenicity islands 1 and 2. B) *Salmonella* nomenclature: The genus *Salmonella* is subdivided into species, subspecies and serovars in accordance with the current taxonomy. The star indicates that only a few selected serovars are given as examples. The *Salmonella* strain used in this work is highlighted in bold and coloured in red.

Each subspecies is further classified serologically in accordance with the White-Kauffmann-Le Minor typing scheme, based on the somatic (O), surface (Vi) and flagellar (H) antigens. To date more than 2,610 serovars have been identified and this is regularly updated by the WHO as new serovars are still being discovered every year (Guibourdenche et al., 2010). Serovars belonging to the subspecies *Salmonella enterica* are usually designated by a name related to their geographical origin (*S. Dublin*), associated disease (e.g. *S. Typhi*) or host specificity (*S. Abortusovis*) (Grimont and Weill, 2007). These names are written

in non-italicized Roman letters with the first letter being capitalized e.g. *Salmonella enterica* subsp. *enterica* serovar Typhimurium, or short as *S.* Typhimurium. All other subspecies are designated by their antigenic formula. The reason behind this nomenclature is that 99.5% of isolated *Salmonella* strains belong to the subspecies I and are responsible for almost all *Salmonella* infections in humans and warm blooded animals (Desai et al., 2013, Grimont and Weill, 2007). In order to detect epidemical outbreaks as well as source attribution, scale and transmission of *Salmonella*, phage typing has been demonstrated to be a successful tool (Baggesen et al., 2010, Miller et al., 2013).

1.2.2 Characteristics of Enterobacteriaceae

The Enterobacteriaceae family include many pathogenic as well as commensal species (e.g. *Salmonella enterica* and *E. coli*), which are Gram-negative, aerobic or facultative anaerobic rods that are non-sporulating, glucose fermenting and oxidase negative (Kumar, 2012). Most species are motile by peritrichous flagella and are able to use nitrate as an alternative energy source when oxygen levels are low (Jones et al., 2011). *Salmonella* and *Escherichia* are closely related species with a genomic hybridisation of 50% (Madigan, 2008), but they can be phenotypically differentiated when compared under the same conditions. The homology between *S.* Typhimurium LT2 and *Escherichia coli* K-12, based on the coding sequence, is 80% (McClelland et al., 2001, Anjum et al., 2005).

The pathogenic *Salmonella* Typhimurium is a common source of food poisoning, whereas many *E. coli* stains form an essential part of the gut flora by suppressing the growth of harmful bacteria and by helping humans to synthesize vitamin K from undigested material in the large intestine; although pathogenic *E. coli* stains exist as well (Ramotar et al., 1984). However, even commensal *E. coli* can become pathogenic when introduced into tissues outside the intestinal tract, causing urinary tract infections, septicaemia, pneumonia or meningitis (Kaper et al., 2004, Tenailon et al., 2010). In comparison, *Salmonella* species are invasive pathogens that can cause a self-limiting

gastroenteritis, which is able to progress into a life-threatening bacteraemia infection in humans (non-typhoidal *Salmonella*, e.g. *S. Typhimurium*) or an often deadly typhoid fever infection (*S. Typhi* and *S. Paratyphi*). Both, *Salmonella* and *E. coli* have a wide temperature range with an optimum of 37°C, which allows them to colonize diverse environments. In addition, they have the ability to survive and adapt to a broad range of stresses, which will be discussed in more detail in following sections.

Besides the fact that the intestine is their prevalent habitat, enteric bacteria colonize the healthy gut in only low concentrations ($<10^8$ cfu/g) compared to the very diverse microbiota of more than 10^{12} bacteria/g (Stecher et al., 2012). Although *E. coli* is outnumbered in the intestine by anaerobic bacteria by a factor of 100 - 1000, it is one of the first bacterial species to colonize the intestine during infancy (Penders et al., 2006, Berg, 1996). Furthermore, triggered by a host's immune response or infection, enterobacterial colonization is boosted and suppresses the anaerobic microbiota during inflammation (Stecher et al., 2007). This same group demonstrated a few years later that gut inflammation increases horizontal gene transfer (HGT) between pathogenic and commensal Enterobacteriaceae (Stecher et al., 2012). Unlike other coli forms, *E. coli* can only survive within the host intestine and dies after a few days of exposure to the environment when excreted with the faeces. Thus, its presence in the food or water supply is indicative for faecal contamination (Kumar, 2012).

1.2.3 *Escherichia coli*

As mentioned in section 1.2.2, most *E. coli* strains are harmless and form an essential part of the normal gut flora. However, some *E. coli* species have acquired certain virulence traits via HGT that enable them to cause diseases including urinary tract infections (UTI), gastroenteritis and meningitis in otherwise healthy hosts (Croxen and Finlay, 2010, Stecher et al., 2012). Expression of virulence factors, such as adhesins, toxins or invasins determine the disease caused and are used alongside the associated serotype, determined by the O (Lipopolysaccharide, LPS) and H (flagellar) antigens, for strain identification (Kaper et al., 2004). Human infections caused by pathogenic

E. coli occur mainly through ingestion of contaminated food and water, contact with people or animals and potentially through airborne transmission (Varma et al., 2003). There are eight common pathovars (pathogenic groups) associated with human diseases that have been extensively studied and that can be classified as either intestinal *E. coli* (enteric or diarrhoeagenic) or extraintestinal *E. coli* (ExPEC) (Russo and Johnson, 2000). Six pathovars are diarrhoeagenic – enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). The two extraintestinal *E. coli* pathovars are associated with UTIs caused by uropathogenic *E. coli* (UPEC) and neonatal meningitis/sepsis caused by neonatal meningitis *E. coli* (NMEC). Other human pathogenic *E. coli* have been isolated, including the necrotoxicogenic *E. coli* (NTEC) or the adherent invasive *E. coli* (AIEC), but their pathogenesis mechanisms are less well understood (Croxen and Finlay, 2010). Although there is some overlap between the different pathovars, such as a type III secretion system (T3SS) that is used to translocate virulence factors (effectors) directly into host cells, they possess a distinct combination of virulence traits that result in diverse pathogenic mechanisms. It has been shown that secretion of these effectors can be greatly enhanced by the presence of nitrate, for anaerobic respiration (Ando et al., 2007). The worldwide burden of diseases caused by pathogenic *E. coli* is increasing and a better understanding of not only the pathovars' mechanisms but also the differences between a commensal and a pathogenic organism will greatly enhance the development of new effective treatments and prevent further epidemics.

1.2.4 Salmonella

In contrast to *Escherichia coli*, *Salmonella* serovars are harmful enteric pathogens that cause morbidity and mortality in both humans and animals and are therefore of economical and clinical importance. The estimated cost associated with *Salmonella* infections in the USA alone is \$2.6bn (Herrick et al., 2012). Nonetheless, *Salmonella* species can also be found as part of the normal flora of poultry and pigs (Humphrey, 2006). In Humans, *Salmonella*

infections vary in symptoms and severity, ranging from self-limiting gastroenteritis (food poisoning) and chronic asymptomatic carriage, which are usually caused by non-typhoidal *Salmonella* (NTS), to systemic typhoid fever (also known as enteric fever) infections caused by *S. Typhi*, *S. Paratyphi* or *S. Sandai* (Runkel et al., 2013). The primary infection route is the ingestion of contaminated food and water, such as eggs and poultry, but there has been an increasing amount of fresh produce-based outbreaks reported over the last few decades (Fatica and Schneider, 2011). Self-limiting salmonellosis or *Salmonella* enterocolitis, a distinct form of gastroenteritis that is mainly associated with *S. Typhimurium* and *S. Enteritidis*, generally causes diarrhoea, vomiting and abdominal cramps. However, in infants, the elderly or persons with immunocompromising conditions such as HIV and cancer, it can progress into an invasive disease like bacteraemia (Okoro et al., 2012). Whilst *S. Typhimurium* causes gastroenteritis in humans, it results in a typhoid-like disease in mice, which makes *S. Typhimurium* an ideal model organism for *Salmonella* research.

Typhoid fever is a major problem in developing countries and requires antibiotic treatment for this otherwise fatal disease. The emergence of multi drug resistant *Salmonella* is a big concern and requires intense research for new treatments and a better understanding of virulence factors and pathogenic mechanisms (Sjolund-Karlsson et al., 2010). *Salmonella enterica* serovars share many virulence factors, some of which are clustered in specific regions on the chromosome, called *Salmonella* pathogenicity islands (SPIs). More than ten SPIs, that are horizontally acquired, have been identified to date. They are involved in invasion, intracellular survival, replication, and host response processes and SPI-1 and SPI-2 are among the best studied (Que et al., 2013). Both, SPI-1 and SPI-2 encode for a T3SS and are crucial for virulence (Srikanth et al., 2011, Fields, 1986). While SPI-1 is required for invasion of epithelial cells, SPI-2 is essential to cause a systemic infection and for intracellular survival by the formation of a *Salmonella* containing vacuole (SCV, protective coat against the host immune response) (Hansen-Wester and Hensel, 2001, Haraga et al., 2008). Furthermore, SPI-2 is thought, somewhat controversially, to protect *Salmonella* from reactive oxygen species (ROS) and reactive nitrogen species (RNS) by preventing the co-localization of the NADPH oxidase Phox and the

inducible nitric oxide synthase (iNOS) with the SCV (Chakravorty, 2002, Vazquez-Torres et al., 2000b). The stress responses inflicted by the host immune response will be discussed in more detail in the following sections.

1.3 Enteric bacteria and anaerobic metabolism

The varied lifestyles of enteric bacteria require metabolic flexibility for rapid adaptations and to ensure survival in diverse environments. The respiratory flexibility of bacteria is distinct from many organisms due to their ability to use a wide range of electron acceptors. These include elemental sulfur and oxyanions, nitrogen oxides and oxyanions, organic sulfoxides, radionuclides, organic N-oxides, transition metals containing minerals and halogenated hydrocarbons (Richardson, 2000). This flexibility has allowed bacteria to colonize many different earth environments of diverse oxygen levels. *E. coli* and *Salmonella* generally use oxygen for aerobic respiration, which is performed by the two membrane-bound quinol oxidases cytochrome *bo* (*cyoAB*) and *bd* (*cydAB* and *cydDC*) (Mason et al., 2009). However, successful colonization of microaerobic and anaerobic environments, like the mammalian gastrointestinal tract (GIT), depends on the ability to use alternative anaerobic electron acceptors, nitrate, nitrite, fumarate, dimethyl sulfoxide (DMSO) and trimethylamine-N-oxide TMAO (Jones et al., 2007, Jones et al., 2011, Paiva et al., 2009, Richardson, 2008). These anaerobic respiratory processes are performed by the nitrate reductases (NarG, NarZ and NapDA), nitrite reductases (NirBD and NrfA), the fumarate reductase (FrdA), the DMSO reductase (DmsAB) and the TMAO reductases (TorCA and TorYZ). The synthesis of these terminal reductases is subject to hierarchical regulation and nutrient availability, so that electron acceptors with a greater redox potential are used preferentially (Jones et al., 2011). Jones *et al.* (2011) further showed in *E. coli* that nitrate is the preferred electron acceptor over fumarate to colonize the mouse intestine and that the DMSO and TMAO reductases are unimportant for this. Furthermore, certain genera of Enterobacteriaceae, including *Salmonella*, *Citrobacter* and *Proteus* but not *E. coli*, have another growth advantage in the

gut; the ability to respire tetrathionate (Barrett and Clark, 1987, Hensel et al., 1999, Winter et al., 2010). Nonetheless, nitrate is the preferred electron acceptor during anaerobiosis and suppresses the genes required for tetrathionate respiration (Winter et al., 2010).

1.3.1 Life in the mammalian gastrointestinal tract

The GIT provides a home for billions of microorganisms that are part of the gut flora but also for pathogens that are able to invade and adapt to this versatile milieu. With an average temperature of 37°C, the GIT provides optimal growth conditions for many enteric bacteria. Nevertheless, in addition to the above mentioned oxygen limitation, various other challenges have to be overcome for successful invasion and colonization. A major obstacle is the stomach acid that is part of the innate immune system, preventing pathogens from invading the GIT by creating a usually lethal pH environment. However, some bacteria such as *Salmonella* and *E. coli*, have evolved sophisticated mechanisms to protect themselves against acid stress. The ability to sense and respond to acid stress is a key feature of enteric bacteria, as they experience rapid pH fluctuations across different environments. The neutralophilic *Salmonella* can grow over a wide range of pH conditions and relies on the combined action of its acid tolerance response (ATR) and acid shock response (ASR) to survive the normally lethal pH level (pH 3-4) in the stomach (Foster, 1991). Besides acid stress, enteric bacteria have to cope with a range of other stresses in the human habitat, including the detergent-like activity of bile, decreased oxygen concentrations, competition with members of the gut flora for binding sites and nutrients, antimicrobial peptides and antimicrobial molecules such as ROS and RNS. *Salmonella* encounters nitrosative stress either during an attack from host macrophages or by their own metabolism during anaerobic nitrate respiration. The mechanisms involved in nitrate respiration and nitrosative stress responses will be discussed in the following sections, with the main focus on the potent cytotoxin nitric oxide and its detoxification product, nitrous oxide. For further information on other *Salmonella* stress responses, interested readers are referred to two comprehensive reviews (Runkel et al., 2013, Spector and Kenyon, 2012).

1.4 Nitrate respiration in *S. Typhimurium* and *E. coli*

This section describes the nitrogen cycle with respect to nitrosative stress, nitrate respiration, denitrification and host-bacteria interactions. It focuses particularly on nitrate respiration and its link to the pathogenicity and survival of two members of the Enterobacteriaceae family of Gamma-proteobacteria in the human GIT, and provides an overview of the mechanisms and regulatory processes involved. Nitric oxide and nitrous oxide are two important products of these processes and form the basis of this work. Unless specified otherwise, all systems and processes described reflect the current understanding of nitrate respiration in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*.

1.4.1 Overview of nitrosative stress

Enteric bacteria such as *S. Typhimurium* and *E. coli* encounter nitrosative stress throughout each stage of their lifecycle in the host. Physical and chemical host barriers of the innate immune system normally protect the host from invading pathogens by activating macrophages, a special type of phagocytes, to engulf and destroy the invaders. Activated macrophages produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are able to modify or inactivate proteins, lipids and nucleic acid compounds of the engulfed microorganism and thereby kill them (Cherayil and Antos, 2001). Reactive nitrogen species, such as the potent cytotoxin NO, are lethal to the majority of bacteria and hamper their intracellular survival. NO reacts with a broad range of targets, resulting in tremendous effects on the cells signaling pathways, gene transcription, regulation processes, cell metabolism and respiratory activities (Husain et al., 2008, Mason et al., 2009, Henard and Vazquez-Torres, 2011). NO-mediated bacteriostatic and bacteriocidal effects are either a direct consequence of reactions of NO with its target or indirectly by promoting the formation of even more potent RNS, RNI and other free radicals like peroxyxynitrite (Hyduke et al., 2007). Other origins of NO include non-specific chemical reactions, product of the organisms' metabolism and product of bacteria sharing an ecological niche (Filenko et al., 2007). Consequently,

enteric bacteria must defend themselves from a range of possible NO sources. They have evolved a suite of mechanisms to gain sufficient protection against nitrosative stress including NO scavengers, detoxification enzymes and enzymes involved in the repair mechanisms. *Salmonella* obtains additional protection via coating by the SCV, which enables it to live inside macrophages. Each route of NO production will be discussed in the following sections, with special focus on bacterial NO generation during nitrate respiration.

1.4.2 NO characteristics and reactivity

NO is a highly reactive, water soluble free radical and with a molecular weight of 30 g mol^{-1} the smallest biological molecular mediator (Fang, 1997). Its small size and lipophilic character allows diffusion across cell membranes, where it reacts readily with diverse targets. Targets of RNS and NO include metalloenzymes, thiol groups, DNA, glutathiones, iron centers and ROS such as superoxide anion (O_2^-) (Poole, 2005, Wink et al., 1991). The soluble guanylate cyclase is another target that becomes activated by NO, producing cGMP, which modulates many cellular activities (Arnold et al., 1977). The reactivity of NO is triggered by the unpaired electron that interferes with molecular targets. Although the correct nomenclature is NO^\bullet , it is commonly presented as NO. The same nomenclature is used in this work. Since NO research became important for immunology in the mid-1980s and was named molecule of the year in 1992 (Culotta and Koshland, 1992), it has become apparent that NO plays many important roles in biological systems, both beneficial and adverse. NO can act as a vasodilator, modulating blood flow in the cardiovascular system, as an intracellular and neuronal messenger or as a cytotoxic mediator in host defence. Furthermore it is an obligate intermediate during denitrification (Fang, 1997, Kim et al., 1999, Poole, 2005, Spiro, 2007). Generally, NO has a very short half-life time of less than a second but it can rise up to an hour depending on the environment and the presence of oxygen or oxygen radicals (Beckman and Koppenol, 1996). Although NO is reported to possess bacteriostatic and cytotoxic effects, it was controversial for a long time whether these effects result from NO itself or are a consequence of RNS, formed during reactions between NO and other free radicals (Arkenberg et al.,

2011). The study of Brunelli *et al.* (1995) is often referred as negative evidence and states that most of the toxic effects are caused by RNS other than NO (Brunelli *et al.*, 1995). Nonetheless, later studies have clearly proven cytotoxic and cytostatic effects of NO under both, aerobic and anaerobic conditions (Gardner and Gardner, 2002, Weiss, 2006, Richardson *et al.*, 2006).

Furthermore, it can be distinguished between direct and indirect effects of NO as well as between NO and nitrosative stress causing reagents, such as S-nitrosoglutathione (GNSO) (Hausladen *et al.*, 1996). Common NO and RNS targets comprise iron sulphur [Fe-S] clusters, hemes and thiols of important metabolic enzymes like cytochrome oxidases *bd* and *bo*, which leads to a respiratory growth arrest (Stevanin *et al.*, 2002, Mason *et al.*, 2009, Richardson *et al.*, 2011). [Fe-S] clusters are crucial components of many regulators, including the ferric uptake regulator Fur. Fur forms iron-nitrosyl complexes with NO, resulting in derepression of iron regulated gene transcription and inhibition of other important regulators (Vine *et al.*, 2010, D'Autreaux *et al.*, 2002). Indirect NO effects are mediated through generation of RNS from the interaction of oxidative and nitrosative compounds. When NO collides with superoxide anions (O_2^-), they react instantly with each other to form the even more reactive peroxynitrite ($ONOO^-$) (Pacher *et al.*, 2007, Beckman and Koppenol, 1996). Peroxynitrite can further isomerize to nitrate or it becomes protonated to peroxynitrous acid (HONOO), which in turn decomposes to the very potent hydroxyl (OH^\bullet) and nitrogen dioxide (NO_2) radicals (Lundberg *et al.*, 2004, Szabo *et al.*, 2007). This reaction is quite common as NO and O_2^- are both generated by activated macrophages and it occurs so fast that it is the only known reaction that is able to outcompete the activity of superoxide dismutase (SOD) (Pacher *et al.*, 2007). For instance, it can block aconitase and fumarase A activity (Keyer and Imlay, 1997, Hausladen and Fridovich, 1994) and interferes with other crucial metabolisms and cell processes including respiration, DNA replication, ribonucleotide reductase activity and the electron transport chain (Husain *et al.*, 2008, Mason *et al.*, 2009, Schapiro *et al.*, 2003, Lepoivre *et al.*, 1991, Wink *et al.*, 1991).

Furthermore, Richardson et al. (2011) showed that NO targets several steps in *Salmonella's* tricarboxylic acid cycle (TCA) (also known as the Krebs cycle), which is the second part of cellular respiration, generating energy for cell growth. When exposed to NO, *Salmonella* is unable to synthesize two essential amino acids (methionine and lysine), caused by interfering with LpdA, an essential component of the pyruvate and α -ketoglutarate dehydrogenase complexes (Richardson et al., 2011). NO induced auxotrophy is not unique to *Salmonella* but is also seen in other enteric bacteria. For instance, NO stressed *E. coli* cells developed a transient branched-chain amino acid auxotrophy (Hyduke et al., 2007). The cytotoxic effect of NO against the bacterial amino acid synthesis pathways was antagonized by the DksA-dependent regulation of amino acid biosynthesis and transport in a murine *Salmonella* infection model (Henard and Vázquez-Torres, 2012). Thus, DksA is important for intracellular growth of *Salmonella* in activated macrophages and *dskA* mutant strains are hypersensitive to the antimicrobial activity of NO, but regain virulence in iNOS-deficient mice. The protective function of the DksA metalloprotein is due to its C-terminal zinc finger cysteine residues, as mutations in any of these abolish the defence against nitrosative stress (Henard and Vázquez-Torres, 2012).

1.4.3 NO generation in the host environment

1.4.3.1 Spontaneous NO generation via chemical reactions

As mentioned in section 1.4.1, a non-specific chemical reaction is one way of generating NO. For instance, after a nitrate rich meal, NO is generated from acidified nitrate in the stomach. Approximately 80-85% of the daily European dietary nitrate intake (approximately 31-185 mg) comes from the ingestion of nitrate rich vegetables, such as lettuce, beetroot, spinach and other leafy vegetables (Gilchrist et al., 2010, Gangolli et al., 1994). An estimated 20-28% of the ingested nitrate is secreted into saliva, where it is reduced to nitrite by nitrate respiring bacteria (van Velzen et al., 2008). Gastric nitrate levels were measured to be approximately 100 $\mu\text{mol/L}$ (McKnight et al., 1997). This is an important metabolic process that would not be possible without the help of bacteria, as humans lack nitrate reducing enzymes. Some beverages that are

rich in polyphenols, like red wine, have similar NO boosting effects at acidic pH levels as nitrate rich foods and thus, there are strict regulations on nitrate levels in drinking water in many countries (Pereira et al., 2013). In the stomach, nitrite is protonated to nitrous acid (HNO_2), which in turn is decomposed into different nitrogen oxides including NO_2 , N_2O_3 and NO (Benjamin et al., 1994). Consequently, the concentration of gastric NO increases significantly after dietary NO_3^- consumption (McKnight et al., 1997). Some of the NO produced is oxidized back to nitrate by haemoglobin in the blood and is recycled by converting and circulating nitrate and nitrite through the body, ensuring its availability when needed during infection (Lundberg et al., 2008). Cured meat, which often contains nitrite as a preservative, further contributes to an increase of RNS in the GIT. Furthermore, it has been shown that nitrate and nitrite plasma levels increase significantly during gastroenteritis, resulting in increased gastric NO production (Dykhuizen et al., 1996). In order to avoid toxic effects and to maintain steady levels in the uninfected host, excessive nitrate and nitrite is flushed out with urine (Prior et al., 2009).

1.4.3.2 NO generation in macrophages

Macrophages are phagocytic cells that operate in the innate and adaptive immune response. They are derived from monocytes circulating in the blood and are found in lymph nodes and the spleen where they ingest and destroy pathogens and foreign molecules. Macrophages sense the presence of invading pathogens via Toll-like Receptors (TLRs), binding specifically to various pathogen-associated molecular patterns (PAMPs) which are present on the surface of bacterial cells. The interaction of a TLR with their specific PAMP, such as Lipopolysaccharides (LPS), an outer membrane component of Gram-negative bacteria, triggers activation of multiple signaling pathways that induce pro-inflammatory cytokines (Kawai and Akira, 2010). TLR-4 is of great importance in *Salmonella* infection as it detects and specifically binds to LPS, thereby inducing macrophage activity via a cascade of signaling pathways. The adaptor protein MyD88 has been shown to be essential for this process (Talbot et al., 2009).

These signaling pathways ultimately result in the production of the toxic free radical, NO, by the inducible nitric oxide synthase (iNOS). iNOS is a member of the NOS family, comprising three different isoforms that vary in physiological activity and location, namely iNOS, neuronal NOS (nNOS) and endothelial NOS (eNOS). The brain has been proven to be a rich source of NO synthesis. nNOS was the first synthase to be cloned from brain isolates and is therefore also referred to as NOS1 (Pacher et al., 2007). The second isoform, which is primarily a product of phagocytic cells, mainly macrophages, is induced by bacterial products and proinflammatory cytokines upon infection and is also known as NOS2 or iNOS. Inducible NOS generates large amounts of NO, which is used by macrophages to kill the engulfed bacterium. The cytostatic and cytotoxic effect of NO can also be used against viruses, fungi, protozoa, and tumor cells. Due to the fact that eNOS was the last one to be identified, it is also called NOS3. The nomenclature NOS 1-3 has been introduced because it has been shown that the isoforms exist in a wider range of organs than originally thought. eNOS and nNOS require an intracellular increase in Ca^{2+} in order to be activated. Unlike nNOS and eNOS, iNOS is unresponsive to changes in intracellular calcium concentrations as calmodium is already tightly bound and activated upon synthesis (Cho et al., 1992). Another difference between the three isoforms is that eNOS and nNOS are constitutively expressed, while iNOS is only expressed during infection after induction by inflammatory stimuli.

Activated iNOS catalyses the two step oxidation of L-arginine to L-citrulline and NO, producing N^G -hydroxy-L-arginine as an obligate intermediate (Wang and Ruby, 2011). iNOS expression in macrophages is triggered by different routes. Essential stimuli include a range of microbial products, the transcription factor NF κ B and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin-1 (IL-1), and interferon gamma (IFN- γ) (Cherayil and Antos, 2001, Lahiri et al., 2010). IFN- γ , which is produced by natural killer cells and T helper cells, causes dimerization of the Janus kinase (JAK) protein signaling cascade which leads to the recruitment of STAT proteins. Activated upon phosphorylation, the STAT proteins are then translocated to the cell nucleus,

resulting in an increased expression of the transcription factor, IRF-1, which in turn binds to the iNOS promoter and activates transcription (Prior et al., 2009).

Furthermore, it has been shown that the metal transporter, Nramp1 (SLC11a1), associated with phagosomal membranes is also able to induce iNOS and is crucial during *Salmonella* infection, presumably via disturbances in cytosolic metal ion concentrations (Nairz et al., 2009). In addition, the invasins SipB, SipC, and SipD which are secreted by the SPI-1 T3SS together with the effectors SopE2 and possibly SopE are required in the regulation of iNOS expression (Cherayil et al., 2000). Mutant strains deficient in these effectors did not induce iNOS. The iNOS mediated production of NO is crucial for protection against *Salmonella* infection and can lead to an anti-apoptotic activity in host cells (Alam et al., 2008). Accordingly, *Salmonella* infection in *iNOS*^{-/-} deficient mice results in higher apoptotic cells in the liver (Alam et al., 2008). *Salmonella* mutants lacking SPI-2 have been shown to be highly susceptible to ROS and RNS, resulting in a decreased intracellular survival rate in macrophages (Gallois et al., 2001) and they are attenuated in mice (Vazquez-Torres et al., 2000a).

As mentioned earlier, the NADPH phagocyte oxidase Phox belongs to the host's army against invading pathogens. It has been demonstrated that mice, deficient in producing iNOS, Phox or both enzymes are much more susceptible to *Salmonella* infection compared to wild-type mice, resulting in increased tissue damage of liver and spleen (Shiloh et al., 1999, Mastroeni et al., 2000). Furthermore, the risk of developing a serious infection with normally harmless commensal bacteria is also elevated (Shiloh et al., 1999). Although both enzymes have clearly been proven to contribute effectively to the antimicrobial activity against pathogens, their function is important at different stages of the infection. Whereas iNOS is essential at a later stage of infection, macrophages are crucially dependent on the bacteriocidal activity of *phox* at an early stage of infection (Vazquez-Torres et al., 2000a). It has been demonstrated that *Salmonella* is able to proliferate in *phox* deficient mice as early as 24 hours post infection (Mastroeni et al., 2000). On the one hand, these findings underpin the importance of both enzymes for the immune system to deter invading

pathogens. On the other hand, it highlights the importance of using dynamic processes to control infections caused by pathogenic bacteria.

Although NO is generally involved in preventing infections, high levels of NO can paradoxically promote *Salmonella* colonization (Stecher et al., 2007). Explanations can be found when looking at the normal flora. Increased NO levels are toxic to the gut flora, resulting in less competition for nutrients and more binding sites for *Salmonella*, which is able to survive the attack by employing NO detoxification mechanism. The different NO detoxification mechanisms will be discussed in section 1.4.5. Thus, in order to prevent an overproduction of NO and the resulting toxic effects to host cells, NO production must be tightly regulated. Reaching a certain threshold, NO itself triggers a feedback mechanism that prevents overproduction, whereas low NO concentrations stimulate iNOS activity via NF κ B activation (Tsai et al., 1999, Tripathi et al., 2007).

1.4.4 Endogenous NO production during denitrification processes

Analogous to NO production in mammalian cells, NO is produced endogenously as an intermediate during bacterial nitrite respiration, a part of denitrification. As outlined in previous sections, the human GIT is largely anaerobic and contains relatively high levels of nitrate. Thus, *Salmonella* and other enteric bacteria have adapted to this milieu by using nitrate as an alternative electron acceptor during anaerobiosis (Richardson et al., 2009). During denitrification, NO is produced via the combined action of various nitrate and nitrite reductases and is then further converted into the neuropharmacological agent and greenhouse gas nitrous oxide (N₂O) by NO reductases and other detoxification enzymes (Figure 3).

As *Salmonella* lacks the subsequent conversion of N₂O to dinitrogen (N₂), it is only a truncated form of denitrification (Arkenberg et al., 2011) (Figure 4). Amongst all bacterial kingdoms, NosZ is the yet only known enzyme that is able to conduct the final reduction step of denitrification and is mainly found in soil

bacteria. However, the last reduction step of N_2O to N_2 only makes a minor difference to the bacterium bioenergetically (Richardson et al., 2009). All enzymes involved in nitrate respiration are cofactor dependent, mainly [Fe-S] clusters, molybdenum and copper ions and are discussed individually in the following sections.

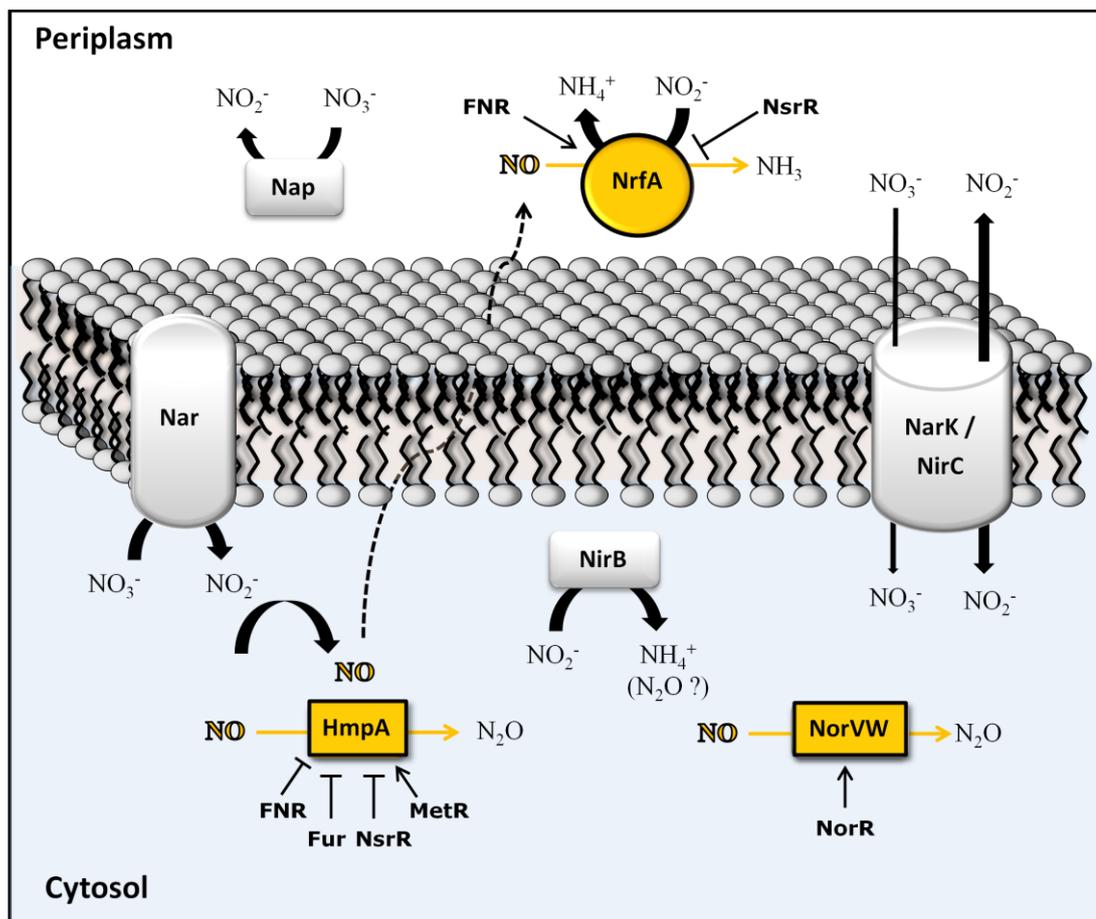
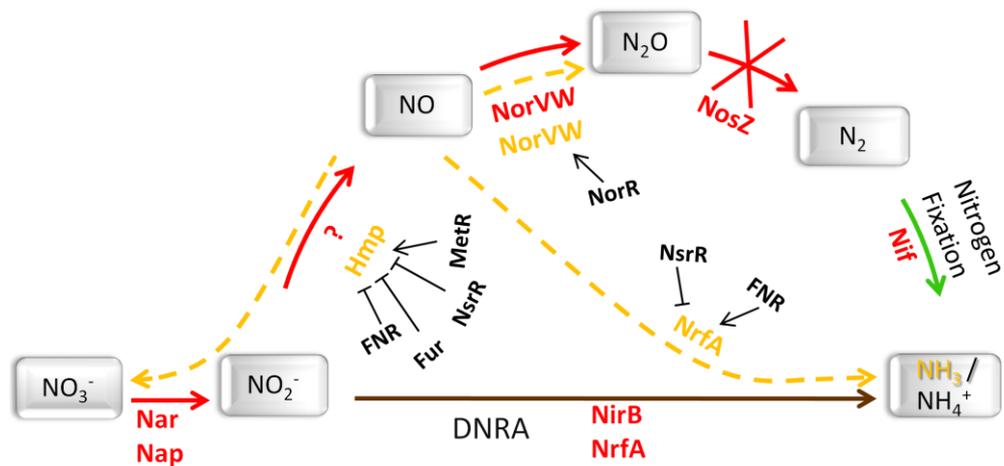


Figure 3 Adapted from Runkel *et al.* (2013). Schematic illustration of the nitrate respiration and NO detoxification pathways of *Salmonella* and *E. coli*.

Important enzymes involved in these processes are shown alongside their respective regulators and cellular location. Positive regulation is highlighted by arrows and negative regulation by perpendicular lines. NO detoxification pathways are highlighted by curved letters and boxes. Endogenously produced NO is able to diffuse across the membrane, indicated by a broken arrow.



Enzymes

Nar membrane-bound dissimilatory nitrate reductase
Nap periplasmic dissimilatory nitrate reductase
NirB siroheme containing nitrite reductase
NrfA cytochrome c nitrite reductase
NosZ nitrous oxide reductase
Nif nitrogenase
NorVW anaerobic nitric oxide reductase flavorubredoxin
Hmp nitric oxide dioxygenase flavohaemoglobin

Regulators

Fur ferric uptake regulator
FNR fumerate and nitrate reductase regulator
MetR methionine repressor
NsrR nitric oxide-sensitive repressor
NorR nitric oxide reductase regulator

Figure 4 Adapted from Arkenberg et al. (2011). Truncated denitrification pathways in *Escherichia coli* and *Salmonella Typhimurium*.

Nitrate respiration in *E. coli* and *Salmonella* is a truncated version of the denitrification pathway (red arrows). Unlike many soil bacteria, *E. coli* and *Salmonella* lack *NosZ*; indicated by a red cross. Nitric oxide (NO) is a toxic intermediate. The main enzymes involved in NO detoxification alongside their regulators are shown. The NO detoxification pathways are indicated by yellow dashed arrows. Enzymes involved in these pathways are shown in yellow. Positive regulation is highlighted by arrows and negative regulation by perpendicular lines. Other enzymes are shown in red.

1.4.4.1 The nitrate reductases Nap and Nar

Salmonella and *E. coli* employ three nitrate reductases to accomplish the first step of nitrate respiration, the reduction of nitrate to nitrite ($\text{NO}_3^- + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$). Most of the initial findings were made in *E. coli* but they equally apply to *Salmonella*, because they both share the same nitrate respiration pathways. *Salmonella* and *E. coli* possess three different nitrate reductases, comprising the periplasmic Nap and the two membrane-bound isoenzymes NarA and NarZ that have their active site in the cytoplasm (Potter et al., 2001, Stewart et al., 2002). NarA consists of the four subunits GHJI encoded by the *narGHJI* operon and uses quinol (e.g. ubiquinol, UQH₂) as an electron transport system to generate a proton motive force (Jepson et al., 2007). NarG, the 150 kDa catalytic subunit of NarA, contains a Mo-bis-molybdopterin guanine dinucleotide (Mo-bis MGD) cofactor with a [4Fe-4S] cluster, a 60kDa [Fe-S] cluster-containing electron transfer subunit NarH and a 20 kDa heme membrane anchor subunit NarI. Furthermore, NarJ is vital for NarA synthesis (Potter et al., 2001, Rothery et al., 2004). NarZ is encoded by the four subunits NarZYWV and shares a 73% homology with NarA (Prior et al., 2009). Nonetheless, the genes encoding the duplicated Nar are differentially regulated and expressed. Under nitrate sufficient conditions, NarA is the most active reductase during anaerobic growth in *E. coli*, whereas the poorly expressed NarZ shows advantages in being active under anaerobic as well as aerobic conditions and during the stationary growth phase (Wang et al., 1999, Potter et al., 1999)

In contrast to Nar, which has been studied since the 1960s, Nap has been the focus of later studies. The structural genes *napFDAGHBC* are contained in the three subunits NapABC. The genes have been sequenced in *E. coli* (Richterich et al., 1993) and were structurally and spectropotentiometrically analyzed (Jepson et al., 2007). Electrons generated from quinol, are transported from the tetra heme cytochrome-c containing NapC, anchored in the cytoplasmic membrane, via the di-heme periplasmic NapB to the catalytic Mo-bis-MGD cofactor and [4Fe-4S] cluster containing NapA (Butler and Richardson, 2005, Nilavongse et al., 2006). Nar is mainly induced under nitrate sufficient

conditions (Rowley et al., 2012, Potter et al., 2001), whereas the nitrate scavenger Nap is the dominant enzyme when nitrate levels are scarce (Constantinidou et al., 2006, Gilberthorpe et al., 2007, Rowley et al., 2012, Wang et al., 1999). The higher expression of Nar indicates that it is the major source of NO production under nitrate sufficient conditions, rather than Nap (Rowley et al., 2012, Gilberthorpe and Poole, 2008). The results are based on transcriptional studies of the genes involved in the NO-responsive NsrR regulon and quantitatively by measuring the end product of NO detoxification, N₂O. The ability of NarG to reduce nitrite to NO was further confirmed with the help of a methyl viologen assay and revealed turnover rates (k_{cat}) of approximately 270 s⁻¹ and 80 s⁻¹ for nitrate and nitrite, respectively (Rowley et al., 2012). Furthermore, it became apparent, that nitrate sufficiency is not the only influencing factor for NO and N₂O production. By creating *nar* and *nap* mutants, it was shown that it is rather a combination of nitrate-sufficiency, nitrite accumulation and an active Nar (Rowley et al., 2012). In nitrate-sufficient continuous culture experiments, a *narG* mutant had a 30-fold lower steady-state rate of N₂O production compared to the WT and a biomass of only 60%, although both of their nitrate consumption rates were comparable (Rowley et al., 2012). In contrast, a *nap* mutant had a steady-state rate of N₂O production that was similar to that of the WT. Additionally, it has been shown that *E. coli narG* mutants have colonization defects in the mouse intestine (Jones et al., 2011). Although Nap and Nar have different roles in response to nitrate, they are both activated by the global regulator FNR and are further controlled by the NarX-NarL and NarQ-NarP two component regulatory systems (Stewart et al., 2009). All regulatory systems, active in denitrification and NO detoxification will be discussed in section 1.4.5.4.

1.4.4.2 NO generation during nitrite reduction

Nitrite generated by Nap and Nar can be further reduced by one of two different pathways; denitrification and nitrite reduction to ammonia. During the denitrification process, *Salmonella* and *E. coli* produce NO as an obligate intermediate, which is quickly reduced to N₂O by the nitric oxide reductase (NorVW) (Figure 4). The second pathway, ammonification, where NO is

generated at low concentrations as a by-product, is conducted by the two distinct nitrite reductases NirB and NrfA that generate ammonia in the cytoplasm and periplasm, respectively (Spiro, 2007). The cytoplasmic, soluble siroheme-containing NirB that is the large subunit (92 kDa) of the *nirBCD* operon uses NADH as an electron donor (Wang and Gunsalus, 2000, Potter et al., 2001). The membrane associated cytochrome c nitrite reductase Nrf got its name from its main electron donor, formate, and is encoded by the *nrfABCDEFG* operon. NrfA has been structurally and spectropotentiometrically characterised and has been shown to be able to detoxify NO to ammonium in addition to its nitrite reducing power to ammonia (Mills et al., 2008, van Wonderen et al., 2008, Clarke et al., 2008b). Its detoxification ability will be further discussed alongside other *Salmonella* NO detoxification mechanisms in section 1.4.5.

The *nirBCD* and the *nrfABCDEFG* operons are regulated by FNR and the NarXL and NarQP two component systems in a similar manner to the nitrate reductases Nar and Nap (Tyson et al., 1993, Wang and Gunsalus, 2000). Nap and Nrf are co-regulated and together represent the periplasmic pathway for the reduction of nitrate, via nitrite, to ammonia. In the cytoplasm, this process is accomplished by the Nar-NirB pathway (Lundberg et al., 2004). Wang and Gunsalus (2000) examined steady-state expression of *nrfA-lacZ* and *nirB-lacZ* reporter fusions in anaerobic *E. coli* chemostat cultures under different nitrate conditions. They aimed to show if and to what respect different nitrate concentrations have an impact on the operons' expression. The findings reveal that NrfA is preferentially expressed at low nitrate concentrations, whereas NirB is maximally expressed under high nitrate conditions (Wang and Gunsalus, 2000). The process of nitrate and nitrite reduction and the proteins involved, are depicted in Figure 3.

Although, the exact mechanism of how NO is generated during these nitrate and nitrite respiration pathways is not completely understood, it has been shown that NarG is the major source of NO production by reducing nitrite (Rowley et al., 2012, Gilberthorpe and Poole, 2008). Furthermore, Rowley *et al.* (2012) showed that anaerobic *Salmonella* cultures, grown under nitrate

sufficient conditions, convert approximately 20% of the consumed nitrate to NO and hence N₂O. This is a considerable amount of intracellular NO and thus *Salmonella* requires sufficient protection mechanisms against the cytotoxin.

1.4.4.3 The role of NarX-NarL and NarQ-NarP in anaerobic nitrate and nitrite reduction

A lot is known about the NarXL and NarQP two component systems and their regulatory function during anaerobiosis in *E. coli*. The *narL* gene, which is located closely upstream of the *narGHJI* operon, was the first gene studied. Shortly afterwards, the nearby *narX* gene was found due to genetic characterization studies of *narL* (Kalman and Gunsalus, 1989, Stewart et al., 1989) and a link to two component systems was drawn (Egan and Stewart, 1990). *narX* and *narL* are transcribed in the same direction and share an eight base pair overlap in their sequence; the *narL* coding sequence comprise the stop codon of *narX* (Egan and Stewart, 1990). The sensor NarX is similar to histidine protein kinases and activates the response regulator NarL by protein phosphorylation in the presence of nitrate. The activated NarL then binds upstream of *narGHJI* and regulates transcription. Egan and Stewart (1990) suggested that NarX alone does not have an essential role in nitrate regulation but rather decreases regulation indirectly by inhibiting *narL* expression.

Homologous to NarXL is the NarQP regulatory system. NarX and NarQ sense nitrate and nitrite and control the phosphorylation of the response regulators NarL and NarP, which results in differential regulation of target operons. The phosphorylation is important for increased binding affinity of NarP and NarL to their specific target DNA binding site, which comprises heptamer sequences arranged in 7-2-7 motifs (Stewart and Bledsoe, 2003). NarXL plays a major role in nitrate respiration by activating *narG* expression (Jones et al., 2011, Stewart and Bledsoe, 2005, Cole, 2012), whereas NarQP has rather a minor role and is primarily involved in activating the periplasmic nitrate reductase pathways via NapA and NrfA (Jones et al., 2011). Furthermore, transcriptional analysis in *E. coli* revealed direct or indirect activation of 51 operons and repression of 41 operons involved in anaerobiosis by NarL whereas NarP induces 14 operons

and represses 37 operons (Constantinidou et al., 2006). Strongly activated operons by phosphorylated NarL include *narGHJI*, *fdnDHI*, the transport protein *narK*, *nirB*, the DNA repair enzymes *ogt* and *ytfE*, *hmpA*, *hcp* and genes of unknown functions *yeaR* and *yoaG* (Constantinidou et al., 2006, Cole, 2012, Karlinsey et al., 2012). All of these are important in their response to NO triggered stress. The study of Constantinidou *et al.* (2006) further showed that NarL is primarily active in the presence of high nitrate concentrations and represses in its absence, whereas NarP activates genes during low concentrations of nitrate and nitrite, but represses in the absence of nitrate or nitrite. The regulation of the *nirBCD* and the *nrfABCDEFG* operons by NarL and NarP differ as well in the presence of nitrate or nitrite. NarL and NarP both activate *nirB* expression if nitrate is present, whereas only NarL is able to do so in response to nitrite. In contrast, *nrf* activation is induced by NarL and NarP by the addition of nitrite. In response to nitrate, NarP activates *nrf* transcription, whereas NarL represses it (Tyson et al., 1993, Wang and Gunsalus, 2000). Both systems have been reviewed in more detail by Potter and co-workers (Potter et al., 2001).

1.4.5 NO protection and detoxification

From the section above, it is clear that enteric bacteria experience a wide range of stresses across their different habitats that require sophisticated protection mechanisms to ensure survival. There are at least four strategies to gain protection from a stress; evasion, preventing the production of toxic substances, acquiring protection by employing a set of detoxification enzymes, or repair mechanisms. For instance, *Salmonella* minimizes its exposure to RNS by preventing lysosomal fusion of iNOS containing vesicles, produced by activated macrophages, with the SCV through the secretion of effectors associated with the SPI-2. Although avoiding lysosomal fusion might not be crucial to prevent contact with NO as it can freely diffuse through membranes, it is important to limit exposure to other RNS that are not able to cross membranes, such as ONOO⁻ (Chakravorty, 2002). The co-localization of the NADPH oxidase (Phox) is prevented in a similar manner (Vazquez-Torres et al., 2000a). Another possibility to prevent the tremendous effect of NO is to use scavengers such as

flavo-haemoglobin (Gardner and Gardner, 2002). Furthermore, the nitrite transporter NirC has been shown to be involved in the inhibition of IFN- γ induced NO production. Thereby NirC assists *Salmonella* in evading macrophage killing and supports intracellular survival and proliferation (Das et al., 2009).

E. coli and *Salmonella* have evolved several NO detoxification mechanisms in order to survive the toxic effects of host-derived nitric oxide and to protect themselves against NO generated by their own metabolism during anaerobic nitrate respiration (Figure 4). NO is converted into the non toxic products nitrate, ammonia and nitrous oxide with the help of three enzymes; the cytochrome c nitrite reductase (NrfA), the flavohemoglobin (HmpA) and the flavorubredoxin with associated NADH-dependent oxidoreductase (NorVW) (Crawford and Goldberg, 1998, Mills et al., 2008). The importance of each enzyme varies with different environmental conditions, which gives *E. coli* and *Salmonella* protection against NO in a range of different environments. Furthermore, different regulators sense and respond to NO and regulate transcription of relevant genes specifically to particular conditions (Rodionov et al., 2005). The core NO response regulators in *Salmonella* and *E. coli* comprise the NorR, NsrR, FNR, Fur and MetR regulators, which are strongly dependent on co-factors and metal ions for correct functioning (Figure 3). In addition to the three well known NO detoxification enzymes, several other genes and proteins have been demonstrated to contribute to *Salmonella*'s protection against nitrosative stress and NO. These include the hybrid cluster protein (Hcp-Hcr), YtfE, and genes where little is known about their function, such as *yeaR-yoaG*, *ygbA* and *STM1808* (Bang et al., 2006, Gilberthorpe et al., 2007, Vine et al., 2010, Rodionov et al., 2005, Filenko et al., 2007, Karlinsey et al., 2012). Each enzyme that is important for NO protection and detoxification will be now discussed separately alongside the key regulators involved in these processes.

1.4.5.1 Key enzymes in NO detoxification

HmpA

E. coli and *Salmonella* possess a 44kDa soluble monomeric flavohemoglobin, HmpA, that consists of an N-terminal heme group and a flavin-binding C-terminal reductase. Electrons are transferred from NADH via the flavin domain to the heme group with the aid of the non-covalently attached FAD (Pullan et al., 2007, Hernandez-Urzua et al., 2003, Mills et al., 2001). Due to its ability to catalyze O₂ as well as its preferred molecule, NO (Vasudevan et al., 1991), it can either be described as a dioxygenase (Mills et al., 2008) or as a denitrosylase (Laver et al., 2010). HmpA is the best characterised NO detoxifying enzyme and was the first bacterial globin, whose gene was sequenced (Mills et al., 2001, Vasudevan et al., 1991). HmpA is able to oxidise NO to either nitrate under oxic conditions or to reduce it anaerobically to N₂O in both, *E. coli* (Gardner et al., 1998) and *Salmonella* (Crawford and Goldberg, 1998). Several *E. coli* and *Salmonella* studies showed that the transcription of *hmpA* can be activated by different sources of NO, such as S-nitroglutathione (GSNO) (Crawford and Goldberg, 1998, Poole et al., 1996, Flatley et al., 2005). Purified HmpA is able to reduce NO to N₂O anaerobically (Kim et al., 1999) but this is only a minor role (Mills et al., 2008) because it detoxifies NO only at a rate of 0.1 – 1% compared to its aerobic activity (Vine and Cole, 2011). Due to its involvement in aerobic NO detoxification in *Salmonella enterica* serovar Typhimurium, it contributes to its virulence in mice (Bang et al., 2006, Gilberthorpe et al., 2007, Stevanin et al., 2007). Furthermore, NsrR, also known as YjeB, was shown to be the principal regulator of HmpA under nitrosative stress conditions (Bang et al., 2006, Bodenmiller and Spiro, 2006). Besides NsrR, other negative regulators include Fur and FNR, whereas MetR activates *hmpA* transcription (Poole, 2005, Membrillo-Hernandez et al., 1998). *Salmonella hmpA* mutants have a severe growth defect upon exposure to NO (Karlinsky et al., 2012) and HmpA was shown to help *Salmonella* to survive and proliferate in macrophages (Gilberthorpe et al., 2007, Bang et al., 2006). Similar observations have been made in *E. coli*. Viability assays demonstrated that HmpA protects *E. coli* from NO-mediated macrophage killing (Stevanin et al.,

2007). Nonetheless, *hmpA* regulation must be controlled to avoid oxidative stress, as HmpA is known as potent generator of superoxide anion from O₂ (Membrillo-Hernandez et al., 1996, Wu et al., 2004, Gilberthorpe et al., 2007).

NrfA

NrfA, the periplasmic cytochrome *c* nitrite reductase of *Salmonella* and *Escherichia coli*, reduces NO₂⁻ or NH₂OH to NH₃ through a six-electron reduction. Furthermore, it has been shown that NrfA is involved in NO detoxification processes under anaerobic conditions (Pooock, 2002, Watmough et al., 1999, van Wonderen et al., 2008) and *E. coli nrfA* mutants are more sensitive to NO than wild-type cultures (Pooock, 2002, Clarke et al., 2008a). Costa et al. (1990) were the first to demonstrate that purified NrfA possess NO reductase ability in *Desulfovibrio desulfuricans* (Costa et al., 1990). The *E. coli* NrfA protein is a 50 kDa pentaheme *c*-type cytochrome which gets electrons from quinol oxidation, catalyzed by NrfD via the subunits NrfC and NrfB (van Wonderen et al., 2008). NrfA was initially believed to reduce NO at a similar rate than NorVW or HmpA (Pooock, 2002), however a later study that used protein film voltammetry measurements showed that NrfA has the highest NO turnover abilities (van Wonderen et al., 2008). Its periplasmic location represents a plausible explanation for this because a lot of the NO becomes metabolized before it is able to enter the cytoplasm. The remaining NO will be efficiently removed by the combined action of NorVW and HmpA.

NorVW

NorVW is an oxygen-sensitive NO reductase that reduces NO to N₂O anaerobically (Gardner and Gardner, 2002, Gardner et al., 2002, Gomes, 2002, Mills et al., 2005, Tucker et al., 2010). In denitrifying bacteria like *Paracoccus*, NorBC fulfils the same reaction. NorVW comprises the NADH-dependent flavorubredoxin oxidoreductase, NorW and the di-iron centered flavorubredoxin NorV. Whereas *norW* mutants show an initial growth inhibition upon NO presence but are able to recover after some time, the anaerobic growth of a *norV* mutant is permanently impaired (Gardner et al., 2002). Transcriptionally

regulated by NorR, *norV* is up-regulated in NO induced *E. coli* cultures (Flatley et al., 2005, Justino et al., 2005b, Mukhopadhyay et al., 2004). Although *norVW* is up-regulated upon macrophage internalization in *S. Typhimurium* (Eriksson et al., 2003), its inactivation does not influence the survival rate of *E. coli* in macrophages and *NorVW* is not required for *Salmonella's* survival in mice (Pullan et al., 2007, Bang et al., 2006).

By comparing all seven possible combinations of *norV*, *nrfA* and *hmpA* single, double and triple *Salmonella* mutants, the importance of each of the three NO detoxification enzymes has been studied under different conditions (Mills et al., 2008). Both, the wild-type (WT) and mutant strains experience a temporary growth arrest upon NO exposure but they differ in their recovery rate. While the WT strain and the *hmpA* and *nrfA* single mutants recovered at similar rates, the *norV* mutant needed more time, suggesting an essential role for NorV in NO reduction. The fact that the *norV* mutant was able to recover at all let assume that other enzymes (NrfA, HmpA or yet unknown enzymes) are able to cover for the loss of NorV activity, even though not as efficiently (Mills et al., 2008). Furthermore, a *norV nrfA* double mutant was unable to recover, which leads to two conclusions: A) NorV and NrfA are *Salmonella's* most important enzymes in anaerobic NO detoxification and B) HmpA is unable to deal with NO on its own under these conditions. These results are consistent with previous *E. coli* studies (Gardner and Gardner, 2002, Hutchings et al., 2002, Pooock, 2002) but are contrary with others (Justino et al., 2005b, Crawford and Goldberg, 1998, Gilberthorpe et al., 2007). These discrepancies might be explained by the use of different growth conditions and NO sources (Mills et al., 2008). With the combined activity of all three enzymes mentioned above, *Salmonella* and *E. coli* are very flexible in their metabolism and well protected against nitrosative stress in a range of different environments. In addition, a recent mouse model study highlighted the importance of HmpA for aerobic NO detoxification by showing that a *Salmonella* strain, lacking a total of 22 genes that are involved in RNS defence had only a slight virulence defect, which could be rescued by a functional HmpA (Burton et al., 2014). This work (Chapters 5 and 6) demonstrates that the enzyme Hcp-Hcr is equally important as Hmp for this process.

1.4.5.2 Enzymes involved in NO metabolism and repair

Although *Salmonella* possesses efficient NO detoxification mechanisms, it additionally employs several NO responsive mechanisms, thought to repair NO induced damage. Genes suggested to be involved in such repair mechanisms include *ytfE*, *hcp-hcr*, *yeaR-yoaG*, and *ogt* (Taverna and Sedgwick, 1996, Vine and Cole, 2011).

Hcp-Hcr

The hybrid cluster protein was first identified in *Desulfovibrio desulfuricans* and it was proposed to possess a [6Fe-6S] cluster, which explains its former name, prismane (Wolfe et al., 2002, Moura et al., 1992). However, later studies have shown that it contains either a [2Fe-2S] or a cubane [4Fe-4S] cluster and the hybrid [4Fe-4S-2O] (Filenko et al., 2007, van den Berg et al., 2000). In *E. coli*, it is highly expressed under anaerobic growth conditions when nitrate and nitrite are available (van den Berg et al., 2000, Filenko et al., 2005, Rodionov et al., 2005) and has therefore been suggested to be part of nitrogen respiration (Wolfe et al., 2002). *E. coli hcp-hcr* is a two gene operon, encoding the iron-sulphur cluster containing *hcp* and its NADH oxidoreductase *hcr*. Initial studies implicated that Hcp has hydroxylamine reductase activity (Wolfe et al., 2002) and that the *hcp* promoter is regulated by FNR, NarL and NarP in response to nitrite and nitrate (Filenko et al., 2005). Although the later statement is still true, doubts arose about the induction of *hcr* by hydroxylamine (Filenko et al., 2007). Microarray studies suggested that Hcp mainly interacts with RNS other than hydroxylamine (Filenko et al., 2007). These findings are supported by *Salmonella* studies where the *E. coli hcp-hcr* homologs *nipAB* and the *ytfE* homolog *nipC* were shown to be up-regulated in activated macrophages, suggesting their impact in defence mechanisms against RNS (Kim et al., 2003). The involvement of Hcr-Hcr in aerobic NO detoxification has been suggested by comparing the respiration and NO reducing activity of a *hmpA* single, a *nsrR hmpA* double and a *nsrR hmpA hcp* triple mutant (Karlinsky et al., 2012).

Further indication for the importance of the hybrid cluster protein in nitrosative stress management is given by *E. coli* mutant experiments, comparing a *nrfAnirBDhmpAnorVW* quadruple mutant with a mutant lacking the *hcp-hcr* genes in addition to the four other genes (Cole, 2012). This study revealed that although the quadruple mutant was able to grow well anaerobically on nitrate, the mutant with all five mutations was unable to grow. Besides this, it has been pointed out that the K_m of Hcp for hydroxylamine reduction to NH_4^+ is higher than the concentration that would completely inhibit *E. coli* growth (Cole, 2012).

Additional experiments, using electrophoretic mobility shift assays, showed that the induction of the *hcp-hcr* operon is totally dependent on anaerobiosis and the regulators FNR and NsrR (Chismon et al., 2010). Other studies showed that *hcp* is repressed by NsrR (Karlinsky et al., 2012, Gilberthorpe et al., 2007, Bang et al., 2006). Although the significance of NsrR in *hcp-hcr* regulation becomes more and more apparent, the exact physiological relevance of Hcp in nitrosative stress remains to be determined. Another interesting observation was made by Seth et al. (2012), who detected endogenous S-nitrosylation in *E. coli*; a mechanism seen in microbes for the first time. They showed that OxyR is a key regulator in S-nitrosylation and that *hcp* transcription is activated by S-nitrosylation of OxyR during anaerobic nitrate respiration. Macrophage experiments and growth assays, using GNSO, revealed that *hcp* protects against nitrosative stress by limiting S-nitrosylation (Seth et al., 2012). H_2O_2 has also been shown to induce *hcp* transcription (Almeida et al., 2006). Collectively, there is clear evidence that the hybrid cluster protein has an important role during nitrosative stress.

YtfE

YtfE, also known as RIC, has a crucial role in the repair of oxidative or nitrosative stress damaged iron sulphur [Fe-S] clusters of metalloproteins (Constantinidou et al., 2006). Surprisingly, this characteristic remained unknown for a long time, although YtfE was known to be highly conserved and widely spread among species (Bodenmiller and Spiro, 2006). Examples include the YtfE orthologue of *gonococcus*, called DnrN (Cole, 2012) or the *scdA* homolog

of *S. aureus* (Overton et al., 2008). In 2005 and 2006, *E. coli* studies showed that *ytfE* mutants have an increased NO sensitivity and growth impairment during nitrosative stress conditions as well as an increased sensitivity to iron starvation (Justino et al., 2005b, Justino et al., 2006). The same group further demonstrated an increased expression of *ytfE* in *fnr* and *fur* mutants, although no obvious FNR or Fur binding sites were found (Justino et al., 2006). The involvement of YtfE in the repair of oxidative and nitrosative stress-damaged [Fe-S] clusters, including the citric acid enzymes aconitase B and fumerase A has been confirmed by other studies (Justino et al., 2007, Vine et al., 2010). One year later it was shown that YtfE contains an iron-sulphur cluster itself and its di-iron centre has been structurally characterised (Todorovic et al., 2008). Several studies showed that *ytfE* expression is repressed by NsrR and it has been suggested that *ytfE* expression becomes activated by NarL upon exposure to nitrate, nitrite and NO (Filenko et al., 2007, Constantinidou et al., 2006, Overton et al., 2008, Gilberthorpe et al., 2007, Karlinsey et al., 2012). Despite all this knowledge, more research is needed to fully understand the mechanisms behind its role in [Fe-S] clusters repair and consequently its contribution to *Salmonella* infection.

Significant Others

Several genome wide studies, including microarray and qRT-PCR, revealed the importance of genes of unknown function for *S. Typhimurium* growth during nitrosative stress and include *ygbA*, *yeaR-yoaG* and *STM1808* (Bang et al., 2006, Karlinsey et al., 2012, Gilberthorpe et al., 2007, Rodionov et al., 2005). It has been shown that *yeaR* transcription is induced by nitrate and nitric oxide (Constantinidou et al., 2006) and that it is negatively regulated by NsrR (Filenko et al., 2007, Karlinsey et al., 2012). Regulation by FNR remains controversial (Filenko et al., 2007, Constantinidou et al., 2006) but seems to be disproved (Lin et al., 2007). Both *yeaR* and *yoaG* are regulated by NarL in addition to the repression by NsrR (Lin et al., 2007, Karlinsey et al., 2012). NsrR further regulates *ygbA* in *E. coli* (Bodenmiller and Spiro, 2006) and *Salmonella* (Karlinsey et al., 2012, Gilberthorpe et al., 2007). YgbA activity is further repressed by FNR (Karlinsey et al., 2012). The novel gene *STM1808* has been

identified during a detailed transcriptional study (Karlinsky et al., 2012). Its regulation by NsrR has been suggested by computational modelling (Rodionov et al., 2005) and was confirmed recently (Karlinsky et al., 2012). Additionally, *STM1808* mutants have a growth defect upon NO exposure (Karlinsky et al., 2012). Database searches further suggested *STM1808* to be a zinc metalloprotein with a His32 and His82 containing domain, important for tellurite and NO resistance (Marchler-Bauer et al., 2011). In conclusion, new genes contributing to *Salmonella*'s protection against nitrosative stress have been identified, but further studies are required in order to understand their specific mechanisms.

1.4.5.3 Enzymes co-factors and metal ions

Many of the above mentioned proteins depend on co-factors for correct functioning. Co-factors are widely distributed and essential for many pathways of the nitrogen cycle. Common co-factors include organic compounds (flavin, haem and molybdenum) or inorganic metal ions (Mg^{2+} , Cu^{+2+} , Zn^{2+} , Mn^{2+} , $Fe^{2+/3+}$ etc.). Enzymes like Nap and Nar or transcription factors such as Fur, FNR, NsrR and NorR exploit their ability to assist in electron transfer, gene regulation and catalytic and sensory processes (Fleischhacker and Kiley, Lill, 2009).

The oldest, almost ubiquitous and most versatile inorganic co-factors are [Fe-S] clusters and are recognized to play a key role in anaerobic nitrate respiration and NO detoxification of *Salmonella* and *E. coli*. They exist as rhombic [2Fe-2S] and cubane [4Fe-4S] clusters or in a more complex form that contain further metal ions. Biosynthesis of [Fe-S] clusters requires complex machinery. Three different types of [Fe-S] cluster biosynthetic systems have been discovered, namely ISC (iron sulphur cluster), SUF (sulphur assimilation) and recently CSD and *ytfE* (Justino et al., 2007). The [Fe-S] cluster biogenesis starts with the release of sulphur from cysteine or histidine by desulphurase. Furthermore, important components include ISC for iron donation and electron transfer, scaffold proteins (serving as platform), cluster transfer proteins and apoproteins (proteins without bound co-factors). A detailed transcription of the biosynthetic

principal of [Fe-S] cluster is given by Lill (2009). However, as mentioned in the previous sections, these clusters are major targets of oxidative and nitrosative stress compounds, which cause displacement of the iron atoms and thereby inactivate the proteins (Todorovic et al., 2008). In addition, the bioavailability of iron can vary. Thus, to antagonize this problem, many pathogens produce high affinity iron-binding compounds called siderophores during oxidative and nitrosative stress (Wang et al., 2011).

1.4.5.4 Regulators

As previously mentioned, a variety of enzymes fulfil different roles in NO metabolism and their activity is strongly dependent on the environmental as well as on multiple transcriptional regulators that mediate a response to NO. Rodionov *et al.* (2005) showed in a very detailed computational genomic study that the regulatory genes and their targets are substantially conserved in evolution but their networks are very flexible between species. Furthermore, care must be taken when comparing regulatory responses to NO with nitrosating substances such as nitroprusside or S-nitrosoglutathione (GNSO) because their responses can vary extremely and would lead to incorrect assumptions (Spiro, 2007). The main NO response regulators of *S. Typhimurium* and *E. coli* will be discussed in this section.

Fur

The ferric uptake regulator Fur is involved in responses to a range of stresses which perturb iron homeostasis. In *E. coli*, Fur controls the expression of more than 90 genes, which are mainly involved in biosynthesis, storage and transport of siderophores (e.g. *bfr* and *feoB*) but also in nitrosative (*hmpA*) and oxidative-stress response (e.g. *sodB*) (D'Autreaux et al., 2002, Hantke, 2001). It possesses an iron centre which is able to bind two NO molecules (D'Autreaux et al., 2004). The link between the control of iron concentration and NO detoxification was made by the suggestion that Fur was able to act as a transcriptional repressor of the *E. coli* and *Salmonella hmpA* gene (D'Autreaux et al., 2002, Poole, 2005, Spiro, 2007, Crawford and Goldberg, 1998). However,

there are different opinions on how and if Fur represses *hmpA* and thus further research has to be performed. Early studies suggested the repression of *hmpA* by Fur and its derepression in the presence of NO or GNSO (Crawford and Goldberg, 1998, Mukhopadhyay et al., 2004). In contrast, microarray experiments showed little to no activity of Fur in response to NO or GNSO (Bang et al., 2006, Flatley et al., 2005). A possible explanation is made by Spiro (2007) who suggests that the use of different media (minimal vs. rich) has an impact on the iron availability. Fur is inactive under iron replete conditions, suggesting a response only when iron is limited to cells. Further studies demonstrated a weak repressor function of Fur on *hmpA* (Hernandez-Urzueta et al., 2007). In *Staphylococcus aureus*, Fur is derepressed by nitrosative stress (Richardson et al., 2006).

MetR

As mentioned above, *HmpA* is regulated by the methionine repressor (MetR), with its cofactor homocysteine (Hcy). Exposure of Hcy to the nitrosating agents GNSO or nitroprusside results in the formation of a S-nitroso-Hcy complex (Bodenmiller and Spiro, 2006, Pullan et al., 2007). In the absence of its co-repressor, MetR binds next to *hmpA* and up-regulates its transcription (Poole, 2005, Membrillo-Hernandez et al., 1998). The construct of a *metR* mutant supports this model, as activation of *hmpA* by the nitrosating agents was lost in the absence of MetR (Membrillo-Hernandez et al., 1998). However, similar to the global regulator Fur, some controversial studies exist (Mukhopadhyay et al., 2004). Furthermore, some suggestions have been made that MetR has a role in regulating *hmpA* expression in response to GNSO but not in response to NO (Pullan et al., 2007, Spiro, 2007). Less efficient nitrosation of Hcy could be a possible explanation.

FNR

The global fumarate and nitrate reductase regulator (FNR) regulates the expression of genes involved in anaerobic respiration in many bacteria. It contains an oxygen sensitive [4Fe-4S] cluster that is required for DNA binding

and sensing the presence or absence of oxygen (Cruz-Ramos et al., 2002). In the presence of oxygen it changes to the more stable [2Fe-2S] cluster, resulting in a loss of its biological activity (Khoroshilova et al., 1997). The switching mechanism of FNR from oxic to anoxic conditions alongside its conformational changes has been well described (Jervis and Green, 2007). FNR is also involved in regulating *hmpA* by acting as a repressor and thus implications have been made that FNR can sense NO in addition to O₂. NO reacts with FNR, causing its inactivation by forming a dinitrosy-iron complex, which in turn results in a derepression of the *hmpA* promoter (Cruz-Ramos et al., 2002). The repressor activity of FNR on *hmpA* was first shown by Poole *et al.* (1996), demonstrating higher activity of *hmpA* in *fnr* mutants (Poole et al., 1996). FNR further regulates *nrfA*, *hcp*, *nar* and *nap*. Spiro *et al.* (2007) highlighted the paradoxon that FNR positively regulates genes encoded by NrfA, an enzymes that plays a role in NO detoxification, although being inactivated by NO. However, this is consistent with a study of Pullan *et al.* (2007), who confirmed that anaerobic exposure to NO leads to up-regulation of FNR repressed genes and down-regulation of FNR activated genes. As mentioned above, FNR activates Nar and Nap during anaerobic growth, together with the two component regulatory system NarX-NarL and NarQ-NarP (Stewart et al., 2009, Potter et al., 2001, Pullan et al., 2007). Similar to the activation of Nar and Nap, FNR regulates the *hcp* gene together with NarL and NarP (Filenko et al., 2005). Furthermore, studies in *E. coli* propose a master function of FNR in the transition between aerobic and anaerobic growth (Tolla and Savageau, 2010) and a very detailed microarray analysis reassessed genes regulated by FNR (Constantinidou et al., 2006).

NsrR

NsrR is another global regulator, able to sense nitric oxide in a wide range of Gram-positive and Gram-negative bacteria including *E. coli* (Bodenmiller and Spiro, 2006), *S. Typhimurium* (Gilberthorpe et al., 2007), *Bacillus subtilis* (Nakano et al., 2006) and *Neisseria gonorrhoeae* (Overton et al., 2006). NsrR is responsible for repression of *hmpA* transcription, which was found to be the most conserved member of the NsrR regulon (Rodionov et al., 2005). NsrR also

represses other genes, known to be up-regulated by nitrosative stress in *E. coli* and *S. Typhimurium*. These include the *ygbA*, *ytfE*, *hcp-hcr* genes and to a lesser extent *nrfA* (Gilberthorpe et al., 2007, Bang et al., 2006, Efromovich et al., 2008, Bodenmiller and Spiro, 2006, Rodionov et al., 2005, Filenko et al., 2007). Based on a technique called plasmid-mediated repressor titration, Filenko *et al.* (2007) revealed that NsrR controls at least 20 genes in *E. coli*. The periplasmic Nap and Nrf are also under its control, but not their cytoplasmic counterparts. Other studies used chromatin immunoprecipitation and microarray (chip-on-chip) analyses to identify binding sites for NsrR in *E. coli*. Novel binding sites such as *feaR*, *feaB* and *tynA* were identified (Efromovich et al., 2008). A list of the great diversity of functions of NsrR regulated genes is given by (Tucker et al., 2010). Studies by Karlinsey *et al.* (2012) confirmed the previously identified NsrR regulated genes and additionally found the *STM1808* gene with a yet relatively unknown function.

Furthermore, NsrR is a member of the Rrf2 family of transcriptional repressors and it has been suggested that NsrR senses NO specifically via a [2Fe-2S] cluster (Bodenmiller and Spiro, 2006). One reason for this assumption is the great similarity between NsrR and other [2Fe-2S] cluster containing members of the Rrf2 family like IscR or RirA. Tucker *et al.* (2008) demonstrated for the first time that the NO-sensitive [2Fe-2S] cluster is required for DNA binding activity. Nitrosylation of this cluster disrupts the DNA binding site and causes derepression of NsrR regulated genes.

NorR

NorR, previously known as YgaA, is the only known regulator that exclusively responds to NO (D'Autreaux et al., 2004, Gardner, 2003, Mukhopadhyay et al., 2004). YgaA was redesignated due to its regulatory function on NorVW. In *E. coli* and *Salmonella* it activates transcription of the *norVW* genes that detoxify NO to N₂O (Hutchings et al., 2002). The discovery of NorR was made by (Pohlmann et al., 2000) in *Ralstonia eutropha*, which has approximately 40% sequence homology to the *E. coli* NorR (Tucker et al., 2009). Initially it has been speculated that NorR is a heme-based sensor (Gardner, 2005), however

another study showed that NorR contains a mononuclear non-heme iron centre (D'Autréaux et al., 2005). Furthermore, NorR consists of three core domains: an N-terminal regulatory GAF (cGMP-specific and cGMP-regulated cyclic nucleotide phosphodiesterase, *Anabaena* adenyl cyclase and *E. coli* transcription factor FhlA), a central AAA+ domain that interacts with σ^{54} -RNA polymerase to hydrolyze ATP and a DNA binding domain at the C-terminal (Tucker et al., 2009). Bioinformatics studies have suggested that three binding sites of NorR activated genes are highly conserved among species (Rodionov et al., 2005). Further studies confirmed that these binding sites are conserved and located upstream of the *norV* promoter and are all required for successful activation by NorR (Tucker et al., 2004, Justino et al., 2005a, Tucker et al., 2009).

1.5 Thesis overview

1.5.1 Research gap

The bacterial production of the neuropharmacological agent and greenhouse gas nitrous oxide and the cytotoxin NO has been extensively studied in denitrifying soil bacteria, however relatively little is known about their endogenous production and function in enteric bacteria. A better understanding of this and the mechanisms involved will provide new insights into host-pathogen interactions, which might lead to new treatment strategies for *Salmonella* infections and potentially help to reduce global warming.

1.5.2 Aims

The intention of this thesis is to enhance the understanding of nitrate respiration processes in enteric bacteria by comparing the physiological and molecular mechanisms involved in endogenous NO production and detoxification in *Salmonella enterica* serovar Typhimurium and laboratory *Escherichia coli* strains. This thesis particularly aims to:

- Validate that there are differences in N₂O levels between the closely related *Salmonella* and *E. coli* during anaerobic nitrate respiration (Chapter 3)
- Identify the reasons behind differences in N₂O production of *Salmonella* and *E. coli* during anaerobic nitrate respiration (Chapter 3)
- Test the hypothesis that *Salmonella* and *E. coli* possess a nitrous oxide reductase (Chapter 4)
- Test the hypothesis that CueO has a nitrous oxide reductase function under anaerobic nitrate respiration (Chapter 4)
- Investigate the contribution of genes belonging to the NsrR regulon in N₂O production of *Salmonella* (Chapter 5 + 6)
- Determine the role of Hcp in anaerobic nitrate respiration in *Salmonella* (Chapter 6)

2 Materials & Methods

2.1 Materials

Chemicals and reagents used in this study were at least of laboratory standards and were mainly purchased from Sigma Aldrich (UK) or Fisher Scientific (UK), unless specified otherwise. All solutions and media were made with dH₂O, apart from steps involving RNA work and High Performance Liquid Chromatography (HPLC). All solutions, required for RNA work, were prepared using molecular biology grade water that is specifically designed to be nuclease and protease-free (Sigma, W4502). This water is from now on called Sigma water. Analytical grade water (Fisher Scientific) was used for HPLC analysis.

2.2 Bacterial strains

The bacteria studied in this work are *Salmonella enterica* serovar Typhimurium strain SL1344 and *Escherichia coli* K-12 derivatives (Table 1). All genetically modified strains described in this study derive from the parental wild-type (WT) *Salmonella* SL1344 strain or the *E. coli* MG1655 WT strain. Strains relevant to each chapter will be described in the materials and method section of the appropriate chapter.

Table 1 Wild-type strains used in this work

Strains	Genotypes and characteristics	
SL1344	<i>Salmonella enterica</i> serovar Typhimurium, his-, mouse-virulent	(Hoiseth and Stocker, 1981)
MC1000	<i>Escherichia coli</i> , $\Delta(lacIPOZYA)X74 galU galK \Delta(araABC-leu)$	(Lacey et al., 2010)
W3110	<i>Escherichia coli</i> , Prototroph	(Lacey et al., 2010)
MG1655	<i>Escherichia coli</i> , Prototroph	(Lacey et al., 2010)
PD1222	<i>Paracoccus denitrificans</i> , Rif ^r , Spec ^r , enhanced conjugation frequencies	(de Vries et al., 1989)

2.2 Bacterial culture conditions

2.2.1 Media

All media compositions including antibiotic concentrations are described in (Appendix A). The minimal medium, MGN, is supplemented with nitrate and glycerol, functioning as electron acceptor and energy source, respectively. Since the *Salmonella* strain used in this study is a histidine auxotroph, casamino acids are required for growth in minimal medium.

2.2.2 Overnight culture

All strains were aseptically streaked onto Luria-Bertani (LB) agar plates (Appendix A), supplemented with antibiotics as appropriate (Appendix A), using one bead of the Microbank™ minus 80°C freezer stocks and incubated statically at 37°C for 16-18 hours. Bacterial plates were stored at 4°C for a maximum of two weeks. Overnight stationary phase cultures were prepared by inoculating sterile glass universals, containing 10 mL LB broth, with a single colony from a fresh LB plate, using an inoculation loop. The cultures were then incubated at 37°C, 200 rpm shaking, for 16-18 hours.

A *Paracoccus denitrificans* overnight culture was prepared in a similar way; however LB plates as well as cultures contained 25 µg/mL rifampicin and were grown at 30°C for 2 days.

2.2.3 Freezer stocks

Bacterial strains were stored at -80°C, using Microbank™ (ProLab Diagnostics) beads according to the manufacturer instructions. Alternatively, DMSO -80°C freezer stocks were prepared by adding 50 µL DMSO to 1.8 mL of a fresh LB overnight.

2.3 Polymerase chain reaction (PCR)

The polymerase chain reaction was carried out as described in Table 2 and Table 3. Template DNA and RNA consisted of either purified plasmid DNA, chromosomal DNA or purified RNA. PCR was used for quality control (check for DNA contamination) of RNA samples, alternatively or in addition to analysis with an Experion (BIO-RAD) (section 2.16.3). One minute per kilobase-pair (kb) of PCR product was used to determine the elongation time. When the annealing temperature needed adjustment, the New England Biolabs T_m calculator was used for optimisation.

2.3.1 Colony PCR

Chromosomal DNA was prepared by dissolving a single bacterial colony, taken from a fresh overnight streak plate, in 200 µL dH₂O by vortexing and boiling for 5 minutes at 100°C to lyse the cell. 5 µL of this was used as template DNA for the PCR reaction.

2.3.2 PCR product purification

PCR products were purified using a QIAquick PCR Purification KitTM (Qiagen) according to the manufacturer's instructions. Amplified DNA adheres to the silica-gel membrane of the purification column and is washed to remove unwanted interference by unused PCR reaction reagents including salts, enzymes, primers and unused nucleotides. The washed DNA was eluted in 50 µL of Sigma water. The purified DNA products were stored at -20°C until further use.

Table 2 PCR reaction components

Reagent	RNA quality test [μ L]	DNA (+) control	DNA (-) control
BIOMIX (Bioline) 2X	12.5	12.5	12.5
Primer Forward (5'- 3') [20 μ M]	0.5	0.5	0.5
Primer Reverse (3'- 5') [20 μ M]	0.5	0.5	0.5
Template (DNA/RNA)	0.5	5	-
dH ₂ O	11	6.5	11.5
Total volume	25	25	25

Table 3 PCR programme details

Program	Mutagenesis		RNA quality test	
	Temperature [°C]	Time [min]	Temperature [°C]	Time [min]
1) Initial denaturation	96°C	3	94°C	3
2) Denaturation	95°C	0.5	94°C	0.25
3) Annealing	52°C - 58°C*	0.5	58°C	0.334
4) Elongation	72°C	0.5 - 2.5†	72°C	0.167
5) Repeat steps 2-4	29 X		28 X	
6) Final elongation	72°C	10	72°C	3 min

*Annealing temperature varied depending on DNA template and primer sequence. † Elongation time was adjusted to 1 minute per kb PCR product.

2.4 Plasmid purification (Miniprep)

A QIAprep Spin Miniprep Kit (QIAGEN) was used according to the manufacturer's instructions for plasmid purification. Briefly, 10 mL of a LB overnight culture containing cells with the desired plasmid, supplemented with the appropriate antibiotic, was grown as described in section 2.2.2 and harvested by centrifugation at 13,000 x g for 3 min at room temperature. After the supernatant was discarded, the cell pellet was resuspended in 500 μ L of Buffer P1 to remove RNA and to chelate metal ions that could block the column. 500 μ L of the alkaline lysis Buffer P2 were added and mixed by inverting the tube several times. Vortexing should be avoided as it would result in shearing the genomic DNA. This Buffer uses sodium dodecyl sulphate (SDS) and sodium hydroxide (NaOH) for cell lysis. To neutralize the pH, 700 μ L of Buffer N3 were added to the solution and mixed by inverting the tube. The potassium acetate of this buffer precipitates SDS, proteins, cell debris and genomic DNA, leaving the

plasmid DNA intact. The mixture was centrifuged at 13,000 x g for 10 min and the supernatant was applied to the anion-exchange column. All following centrifugation steps were performed at 13,000 x g for 1 min. The column was centrifuged, allowing the plasmid DNA to bind to the column. After the flow-through has been discarded the column was washed with 500 µL of Buffer PB and was then centrifuged. Afterwards the column was washed with 750 µL PE buffer, containing Ethanol (EtOH) to solubilise smaller pieces of left over genomic DNA so that they can be eluted, followed by centrifugation. The centrifugation step was repeated to remove residual EtOH. Finally, the purified plasmid DNA is eluted from the column into a sterile Eppendorf tube by applying 50 µL of dH₂O to the column, allowing it to stand for 1 min, followed by a final centrifugation step. Plasmid DNA was quantitatively analysed using a NanoDrop as described in section 2.9 and stored at -20°C until further use.

2.5 Green plates

Green plates were used after P22 transduction to differentiate pseudo-lysogens from true lysogens and were prepared according to (Maloy et al., 1996) (Appendix A).

2.6 Ammonia testing

Ammonia was colourimetrically tested by the wet chemical method. Two solutions were prepared as described below. All chemicals were dissolved in dH₂O. Solution 1 was prepared by mixing 810 mM sodium salicylate with 440 mM tri-sodium citrate and 3.25 mM sodium nitroprusside in a sterile Schott-Duran bottle. The solution was autoclaved and covered in tin foil for light protection. Solution 2 was prepared by mixing 7.8 mM sodium dichloroisocyanurate with 0.8 M NaOH. It should be noted that the NaOH needed to be dissolved in the fume hood before the sodium dichloroisocyanurate was added due to toxic gas development. It is important to wear appropriate protective cloths while performing this experiment. NH₄CL,

prepared in dH₂O, was used to create a standard curve in the range of 1 µM - 1 mM concentrations. To start the reaction, 800 µL of diluted sample (1:200 worked best for chemostat samples) or NH₄CL was mixed thoroughly with 300 µL of solution 1 and 300 µL of solution 2 in an Eppendorf tube. The mix was stored for 60 min in the dark and the absorbance was measured at 655 nm against a blank, containing dH₂O and both solutions. There is a colour shift from yellow (1 µM ammonia) to dark blue (1 mM ammonia). Outside of this range the colour reverses in both directions. At concentrations below 1 µM the colour changes towards blue via green, whereas at concentrations above 1 mM the colour changes from dark to light blue and would eventually end up in green and yellow.

2.7 Anaerobic batch culture

Batch culture is a closed system where organisms are grown in a fixed volume under certain environmental conditions (e.g. nutrients, pH, and temperature) for only a few generations, until the nutrients are used up or the toxins produced are too high. The advantage of batch cultures is that the experiments can be carried out in a large number in parallel and that they are relatively quick and easy to perform. Growth in a batch culture has four phases: a lag phase, an exponential phase (also known as log phase), a stationary phase and a death phase (Figure 5). As the dilution rate in a batch culture is zero, the specific growth rate (μ) is defined by the following formula.

$$\frac{dx}{dt} = \mu x ; x = \text{biomass}, t = \text{time}$$

During the lag phase, bacteria adapt to the environmental conditions and synthesize relevant enzymes and other molecules. In the exponential phase, bacteria double and achieve their maximal growth rate (μ_{\max}). The growth rate slows down and eventually stops in the stationary phase due to nutrient limitation and accumulation of toxins. Once all nutrients are consumed, bacteria start to die in the death phase. This study uses special batch culture vessels, Hungate tubes, for anaerobic growth that allow gas sampling, using a gas tight syringe, via the air tight septum.

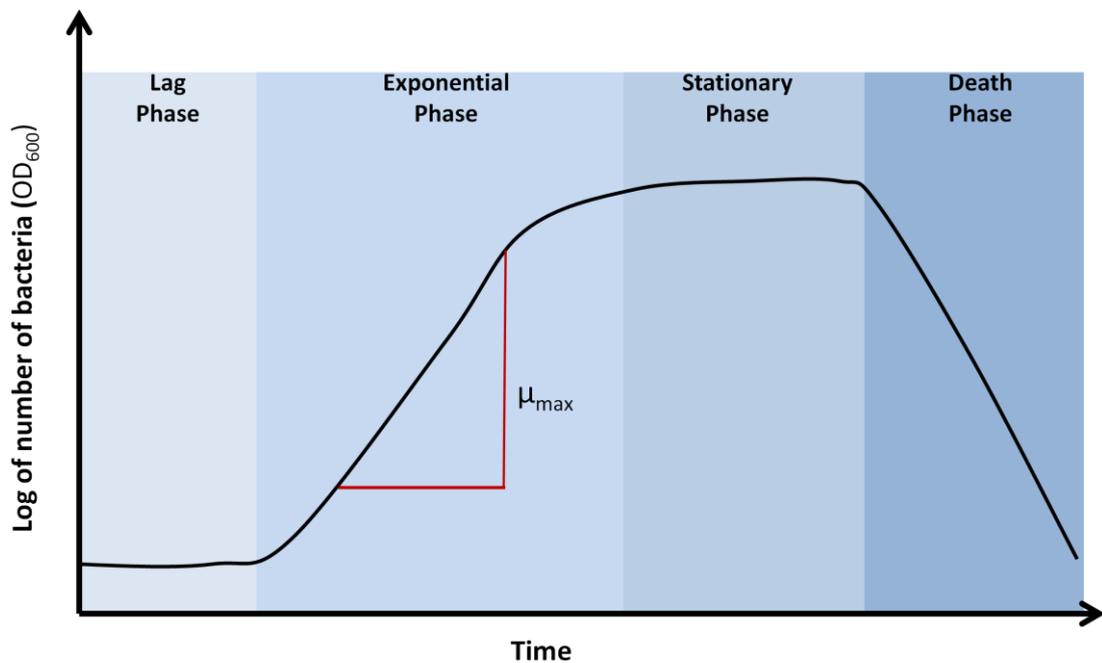


Figure 5 Schematic batch culture growth. μ_{max} is the maximal growth rate.

2.7.1 Hungate batch procedure

The anaerobic batch growth of different *E. coli* and *S. Typhimurium* strains was performed in Hungate tubes (BellCo Glass Inc. 15 mm open top screw cap). A Hungate tube, containing 10 mL of minimal MGN media with appropriate supplements (Appendix A), was inoculated with 2% (v/v) of a 10 mL MGN overnight culture (prepared as described for a LB overnight), using a 0.5 mL Hamilton syringe (Microlitre™) and incubated statically at 37°C for 24 hours. Hungate tubes are autoclaved separately from the media. Autoclaved media was transferred into sterile Hungate tubes in the laminar flow hood. When gas samples were taken from the Hungate batch cultures, it was ensured that the Hungate tube was not shaken or inverted to avoid gas exchange with the liquid phase that would result in a false reading.

2.8 Continuous culture

Continuous cultures are an important tool for microbial physiology research and have several advantages over batch cultures. In contrast to the closed batch systems, where growth ends when nutrients are used up or when the amount of toxic by-products becomes too high, continuous cultures can be run for a longer period of time under the right conditions. A common continuous culture system is the chemostat, a bioreactor which is constantly supplied with fresh medium and where a constant volume is maintained by controlled efflux of culture liquid. By changing the feed/flow rate of the medium the growth rate can be easily controlled. In addition, growth is limited with respect to one substrate, commonly the carbon/energy source but also other nutrient limitations are possible, whereas all others are in excess. Furthermore, other culture parameters (e.g. DO, pH, temperature) are controlled and constantly monitored. One of the most important features of chemostat, where all culture parameters remain constant, is the growth in steady state. Steady state is achieved if the dilution rate (D) equals specific growth rate (μ). The dilution rate (D) is defined as flow rate (F) over the culture volume (V). This feature allows direct comparison of the organisms' metabolites between different runs.

Steady state growth is achieved if:

$$\text{Dilution rate } (D) = \frac{\text{Medium flow rate } (F)}{\text{Culture Volume } (V)} = \text{Growth rate } (\mu)$$

One major drawback is that continuous chemostat cultures are prone to mutations to achieve a competitive advantage. Therefore the chemostats in this study were run for a maximum of one week. The chemostat used for this study was a stir tank bioreactor (Figure 6B). Nutrient limitations were either set for the carbon (energy) source or the nitrogen (electron (e^-) acceptor; substrate for anaerobic nitrate respiration) source.

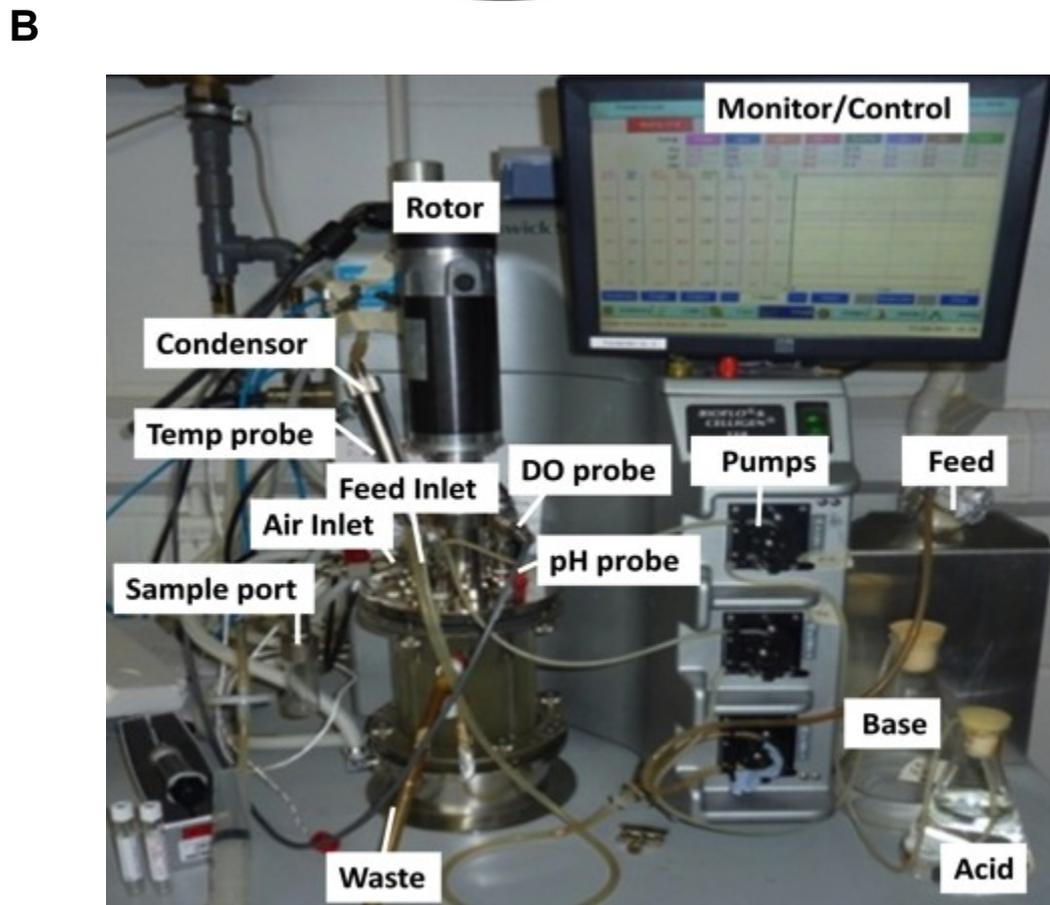
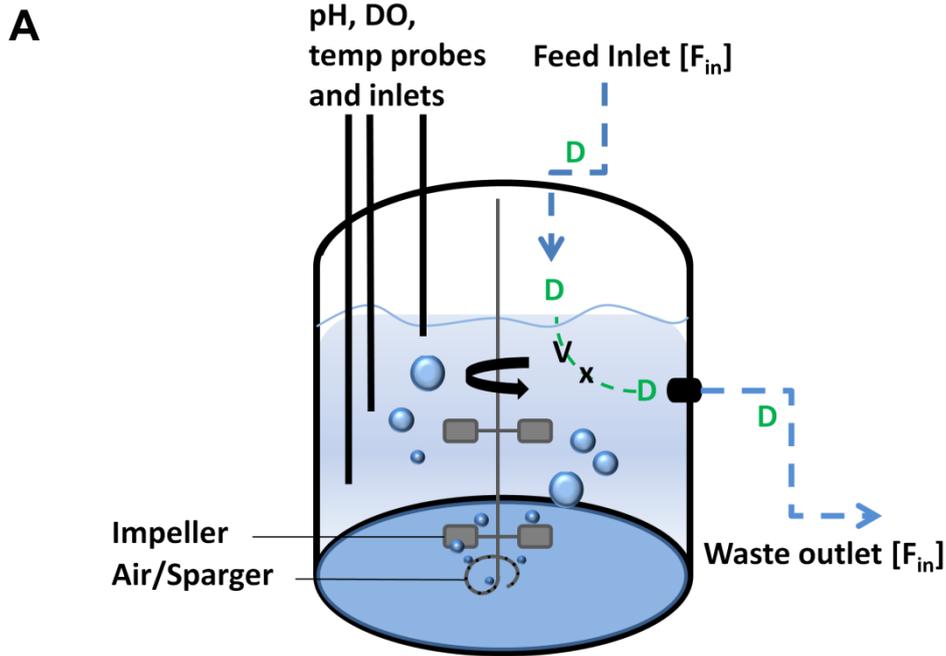


Figure 6 Schematic chemostat (A) and New Brunswick Scientific Bioflow 300 chemostat, used in this study (B).

(A) The most important feature of chemostats is the growth steady state, achieved when the dilution rate (D) equals the specific growth rate (μ), which is dependent on the Flow rate (F), the culture Volume (V) and the Biomass (x). (B) All culture parameters (e.g. pH, temperature, dissolved oxygen) are constantly controlled and monitored. The pH is maintained by adding acid (0.1 M H_2SO_4) and base (1 M $NaOH$). Gas and liquid samples are taken at different times to test the growth (OD_{600}) and the production of different metabolites.

2.8.1 Chemostat procedure

The method was adopted from (Rowley et al., 2012). The bacterial strains used were cultivated anaerobically in MGN medium. Glycerol was used as carbon source and sodium nitrate as terminal electron acceptor. Continuous cultures were grown in 2 L New Brunswick Scientific Bioflow 300 chemostats under pH control (pH 7.5, 1 M NaOH and 0.1 M H₂SO₄ used for regulation) and a constant temperature of 37°C. A Mettler Toledo 405-DPAS-SC-K85/200 was used to monitor the pH. The chemostats were calibrated for pH as well as dissolved oxygen (DO) before each run. Afterwards, the chemostat vessel containing 1.5 L of MGN media was autoclaved. 100 mL MGN medium was inoculated with a 5 mL LB overnight culture and aerobically incubated over night at 37°C. 50 mL of this culture was used to inoculate the bioreactor. The culture was allowed to grow aerobically for 22 h by constant air supply and mixing at 200 rpm to increase biomass. Afterwards, the air supply was cut off and a feed reservoir, containing MGN, was started to achieve a dilution rate (D) of 0.0467 h⁻¹ (D= flow rate [70 mL h⁻¹] / chemostat volume [1.5 L]). The pumps for the feed reservoir were calibrated before each run. The dissolved oxygen (% air saturation) was monitored throughout the experiment by a Mettler Toledo InPro 6800 DO-probe. A minimum of four fermenter volume changes allowed the culture to go into a steady state. During the experiment, gas and liquid samples were taken at regular intervals to determine the OD₆₀₀, nitrate, nitrite, N₂O concentrations and RNA levels. Liquid samples were transferred to 1.5 mL Eppendorf tubes and centrifuged at 13780 x g (13,000 rpm). The supernatant was transferred into fresh tubes and stored at minus 20°C until further use for HPLC analysis, whereas cell pellets were stored at minus 80°C for later RNA extraction. Gas samples were taken as described in section 2.11.1

2.9 Spectrophotometry

For measurement of bacterial growth, 250 mL conical flasks, containing 100 mL LB broth were inoculated with 1:100 (v/v) of a LB overnight culture and grown at 37°C, 200 rpm shaking, for up to eight hours. At 60 min intervals, 1 mL culture was aseptically transferred to a 1.5 mL plastic cuvette and the optical density at 600 nm (OD_{600}) was measured using a spectrophotometer (Molecular Devices, SpectraMax M5). One millilitre sterile LB broth was used as reference. Exceeding an OD_{600} value of two, the culture was diluted in a 1:10 ratio with dH_2O . The dilution factor was considered when calculating the OD_{600} value.

A NanoDrop 2000 (Thermo Scientific) was used according to the manufacturer instruction to determine DNA and RNA concentrations. One microlitre of sample was placed onto the pedestal and measured against Sigma water, used as a blank. The ratio of absorbance at A_{260}/A_{280} and A_{260}/A_{230} is used to assess the purity of DNA and RNA. As a guide, a ratio of approximately 1.8 indicates pure DNA and a ratio of approximately 2.0 or above indicates pure RNA for the A_{260}/A_{280} ratio. Values lower in either case indicate presence of protein and other contaminations. The ratio A_{260}/A_{230} is used as a secondary measure of nucleic acid purity and should result in values between 2.0 and 2.2, if pure.

For growth measurements of anaerobic cultures, a Fisher Scientific spectrophotometer (Calorimeter model 45) was used, which allowed direct measurement (at OD_{590nm}) of cultures grown in Hungate tubes. A Hungate tube, containing 10 mL of MGN media, was used as reference.

2.10 Agarose gel electrophoresis

Agarose gel electrophoresis was used for separation, visualisation and quality control of PCR products. 10 x TBE buffer (106 g Tris base, 55 g boric acid and 40 mL EDTA, made up to 1 L with dH_2O) was diluted 1:10 to make a working solution of 1 x TBE Buffer. A 1% agarose gel (w/v) was prepared by mixing 1%

agarose (w/v) with 1 x TBE and heated up in the microwave until completely dissolved. The solution was allowed to cool down until it was lukewarm, after which 0.04% (v/v) Ethidium Bromide (wear gloves all the time) was added before the agarose solution was poured into a gel cast. A multi-well comb was mounted to create loading wells and the gel was allowed to set at room temperature. Once set, the comb was removed and the gel was transferred to an electrophoresis tank (Sub-Cell GT, BIO-RAD) filled with 1 x TBE buffer. For later visualisation, 5 μ L PCR product was loaded into the wells and the gel was run at 110 V for 20-50 min, depending on the size of the PCR product. A 1 kb Hyperladder (Bioline) was used as marker for size comparison. As the PCR product contains BIOMIX (Bioline), there was no need to add additional loading dye to the sample before running the gel. However, when purified PCR product, plasmid DNA or RNA was used, 1 μ L of 5 x loading buffer (Bioline) was added to 1 μ L of sample and 4 μ L dH₂O. Gels were visualized using a Gel Doc™ XR+ (BIO-RAD) system.

2.11 GC

2.11.1 Sampling and storage of N₂O

Gas samples of 12 mL and 3 mL were taken using a 25 mL gas tight Hamilton syringe (Model 1025 SL SYR (22/2"/2)) and a 5 mL gas tight Hamilton syringe (Model 1005 SL SYR (22/2"/2)) from continuous chemostat cultures and Hungate batch cultures, respectively. The gas samples were transferred into 12 mL and 3 mL pre-vacuumed soda glass Labco Exetainers (839W, 829W). The gas samples were stored at 4°C and allowed to adjust to room temperature before measurements were performed.

2.11.2 N₂O measurement

A Perkin Elmer Clarus 500 Chromatograph with an electron capture detector (ECD) and an Elite-Q PLOT phase capillary column (length: 30m and 0.53 mm inner diameter) was used to measure N₂O gas samples and was run under the

following conditions: BOC gas cylinders; 5% Methane/Argon (make up gas), Zero Nitrogen (carrier gas), 20 split (20% sample go to waste, 80% passing down the column) at 60 psi and temperatures as described by the manufacturer. 50 μL gas samples were injected using a 100 μL gas tight Hamilton syringe (Model 1710 SL SYR (22s/2"/2)). Each sample was analyzed in duplicate. In between each sample, the syringe was flushed a few times with air to ensure complete removal of the previous sample. Care was taken to ensure that sample had atmospheric pressure before injection. N_2O standards (Scientific and Technical Gases (STG) LTD; 0.04 ppm – 10000 ppm) were used to create a calibration curve to convert peak area into ppm. N_2O concentrations [ppm] were further converted to μM (section 2.11.3). N_2O had a retention time of approximately 5.3 minutes. Due to the solubility of N_2O in water, a Henry's law constant of 0.453 was used to account for the N_2O present in the liquid phase (solution) (section 2.11.4).

2.11.3 Conversion of N_2O ppm (by mass) to μM

The concentration of N_2O standards, used to create a calibration curve, was given in parts per million (ppm). Thus, the concentration of N_2O had to be converted in μM levels in order to calculate the nitrogen balance (Nitrate put in the system $[\text{NaNO}_3]$ = nitrogen equivalents $[\text{N}_2\text{O}$, Nitrate, Nitrite, Ammonia], products of nitrate respiration).

The calibration gas standards contain N_2O in N_2 . In order to determine the amount of moles N_2O present in 1 g of $\text{N}_2\text{O}/\text{N}_2$ mixture the following formula was used:

$$\frac{m}{M} = n$$

Where :

M = Molar mass [g/mol]

m = Mass [g]

n = Amount of molecules [mole]

Given:

$$m = 1 \text{ ppm N}_2\text{O} = 1/1000000 \text{ g/g} = 1 \times 10^{-6} \text{ g}$$

$$M_{(\text{N}_2\text{O})} = 44.013 \text{ g/mol}$$

$$\frac{1 \times 10^{-6} \text{ g}}{44.013 \text{ g/mole}} = 2.27 \times 10^{-8} \text{ mole N}_2\text{O}$$

The amount of moles per one gram of N₂ was calculated by the same formula.

Given:

$$m = 1 \text{ g}$$

$$M_{(\text{N}_2)} = 28.013 \text{ g/mol}$$

$$\frac{1 \text{ g}}{28.013 \text{ g/mole}} = 3.57 \times 10^{-2} \text{ mole N}_2$$

Thus, 1 g of N₂O/N₂ mixture contains:

$$\frac{2.27 \times 10^{-8} \text{ mole}}{3.57 \times 10^{-2} \text{ mole}} = \mathbf{6.36 \times 10^{-7} \text{ mole}}$$

As N₂O and N₂ are gases, the volume of one mole of ideal gas at atmospheric pressure was determined by using the following formula:

$$pV = nRT$$

$$V = \frac{nRT}{p}$$

Where:

V = Volume of gas

n = Amount of molecules [mole]

R = Gas constant [8.314510 J K⁻¹ mol⁻¹] or [m³ Pa K⁻¹ mol⁻¹]

T = Temperature of chemostat [310.15 K = 37°C]

P = Partial pressure [101325 Pa = 1atm]

$$V = \frac{1 \times 8.314 \times 310.15}{101325} = 0.0254 \text{ m}^3 = \mathbf{25.4 \text{ L}}$$

The concentration was then converted to μM as follows:

$$\frac{6.36 \times 10^{-7} \text{ mole}}{25.4 \text{ L}} = 2.504 \times 10^{-8} \text{ mole/L} = \mathbf{2.504 \times 10^{-2} \mu\text{M.}}$$

2.11.4 Henry's law constant

As mentioned in section 2.11.2, some of the N₂O produced in chemostat is present in the liquid phase due to its solubility. Thus, in order to determine the total N₂O production (headspace + solution) a Henry's law (Sander, 1999) constant of 0.453 at 37°C [310.15 K] was used to account for the amount of N₂O present in the liquid phase, assuming that there is an equilibrium between the gas and liquid phase.

Henry's law is a gas law that was formulated in 1803. It states that the concentration of a gas that dissolves in a specific volume of liquid is directly proportional to the partial pressure of this gas in equilibrium with the liquid, at a constant temperature. The equations used in this section was obtained from (Sander, 1999). Briefly, The Henry's law constant, k_H , is defined as:

$$k_H \stackrel{\text{def}}{=} c_a / p_g$$

Where:

c_a = concentration of the trace gas in the aqueous phase

p_g = partial pressure of the trace gas in the gas phase

The commonly used unit for k_H is [M/atm] which equals [mol/atm x dm³].

In order to use the constant for comparisons between different experiments it can be expressed as a dimensionless ratio as follows:

$$k_H^{CC} \stackrel{\text{def}}{=} c_a/c_g = k_H \times RT$$

Where:

R: Gas constant [8.314510 J K⁻¹ mol⁻¹] or [m³ Pa K⁻¹ mol⁻¹]

T: Temperature of chemostat [310.15 K = 37°C]

A series of calculations have to be undertaken to determine the k_H^{CC} . Henry's law constant can be described as a function of temperature, where $\Delta_{\text{soln}}H$ is the enthalpy of the solution, k_H^\ominus is the Henry's law constant k_H under standard conditions ($T^\ominus = 298.15$ K and 1atm pressure) and R is the gas constant.

$$k_H = k_H^\ominus \times \exp\left(\frac{-\Delta_{\text{soln}}H}{R} \left(\frac{1}{T} - \frac{1}{T^\ominus}\right)\right)$$

The temperature dependence describes the change in k_H being equal to the enthalpy of the solution in the following equation:

$$\frac{-d \ln k_H}{d(1/T)} = \frac{-\Delta_{\text{soln}}H}{R}$$

The relation between the k_H and k_H^{cc} can be expressed as:

$$T [\text{K}] \times k_H [\text{M/atm}] = 12.2 \times k_H^{cc}$$

$$k_H^{cc} = \frac{k_H \times T}{12.2}$$

With the help of these equations the k_H and k_H^{cc} can be calculated for N_2O under the experimental conditions of 37°C [310.15 K] as follows:

N_2O has a k_H^\ominus of 2.5×10^{-2} M/atm and $\frac{-d \ln k_H}{d(1/T)}$ value of 2600 (data obtained from (Sander, 1999)). Thus, the k_H of N_2O at 310.15 K is calculated as follows:

$$k_H = k_H^\ominus \times \exp\left(\frac{-\Delta_{\text{soln}}H}{R} \left(\frac{1}{T} - \frac{1}{T^\ominus}\right)\right)$$

$$k_H = k_H^\ominus \times \exp\left(2600 \left(\frac{1}{310.15} - \frac{1}{298.15}\right)\right)$$

$$k_H = 0.0178 \text{ M/atm}$$

This value is used to determine the dimensionless k_H^{cc} of N_2O at 310.15 K:

$$k_H^{cc} = \frac{k_H \times T}{12.2}$$

$$k_H^{cc} = \frac{0.0178 \times 310.15}{12.2}$$

$$k_H^{cc} = 0.4525$$

Thus, to determine the N₂O in the solution (liquid phase), the amount of N₂O measured in the gas phase (N₂O_{gas}) has to be multiplied by the Henry's law constant of 0.4525 (at 37°C). The total amount of N₂O produced (N₂O_{tot}) can be determined by the following formula:

$$N_2O_{tot} = N_2O_{gas} \text{ (measured)} + N_2O_{solution} (N_2O_{gas} \times 0.4525)$$

2.12 N₂O Electrode

An oxygen electrode (Hansatech Instruments Ltd.), also known as Clark electrode, was modified to allow N₂O detection. Thus, it could be used as a method to determine if an organism possesses a N₂O reductase (N₂OR). In order to be able to detect N₂O, the platinum cathode - silver anode electrode disc was replaced by a silver cathode – silver anode disc. In addition the control unit was modified to allow a higher polarising voltage of -1.2V that is required for N₂O reduction (Alefounder and Ferguson, 1982). To minimise electrolyte breakdown at the higher polarising voltage, the electrolyte used consisted of 1 M KOH and 100 mM KCl. Although this modified electrode was optimised for N₂O detection it still reacted to oxygen shifts and therefore the system had to be made anaerobic, as described in section 2.12.3, before the experiment was performed.

2.12.1 *Electrode preparation*

The electrode was prepared according to the Oxytherm System Operations Manual with the modifications mentioned in section 2.12 (Figure 7). Before the electrode disc was prepared it was cleaned and polished gently by using a cotton bud, a few drops of dH₂O and electrode polishing paste (Hansatech). This removes the brown silver chloride that forms from crystalline electrolyte during electrode use. Cleaning of the electrode was also necessary after each experiment, before it was stored air tight in a desiccator to avoid oxidation. Preparing the electrode disc, first one drop of electrolyte was placed on the top

of the electrode disc, covering the silver cathode and three drops around the electrode well, touching the silver anode (Figure 7A). Next, an approximately 1.5 square centimetre spacer paper (cigarette paper works best) was placed over the electrolyte, ensuring that at least one end reaches the electrode well. This was covered by a similar sized piece of PTFE Membrane (Figure 7B). A rubber o-ring was pushed over the electrode dome, using an applicator tool, ensuring that the membrane was smooth and no air bubbles were trapped (Figure 7C). Afterwards the electrode well was topped up with a few drops of electrolyte, if needed, and sealed by an outer rubber o-ring. The prepared electrode disc was assembled with the electrode chamber and placed on top of the electrode control unit. The electrode chamber has a 3 mL volume capacity, allows temperature control and has a stopper with a narrow shaft that is used as the injection point and that minimises gas diffusion with the atmosphere. The control unit applies a voltage to the electrode disk and contains a magnetic stirrer unit. A schematic version of the N₂O electrode is given in Figure 8.

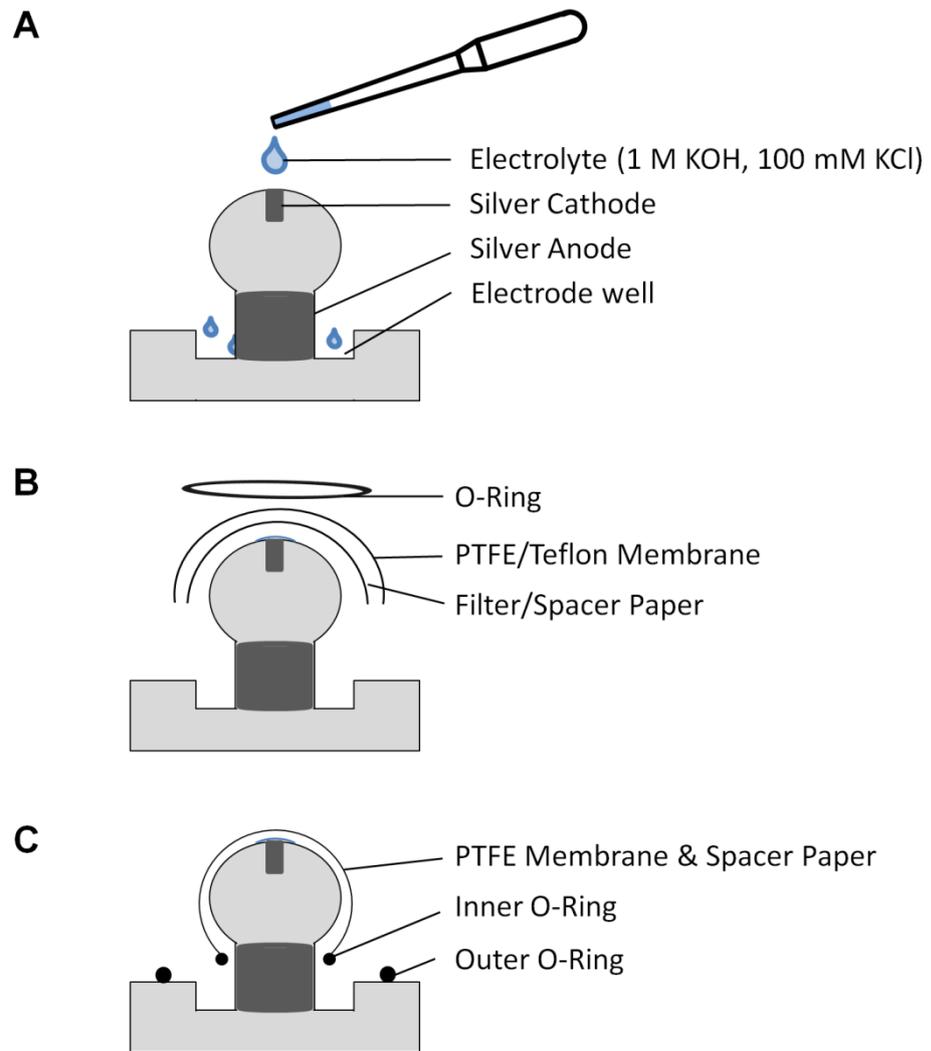


Figure 7 Electrode disc preparation.

An oxygen Clark electrode was modified to allow detection of N_2O using a silver-silver electrode disc.

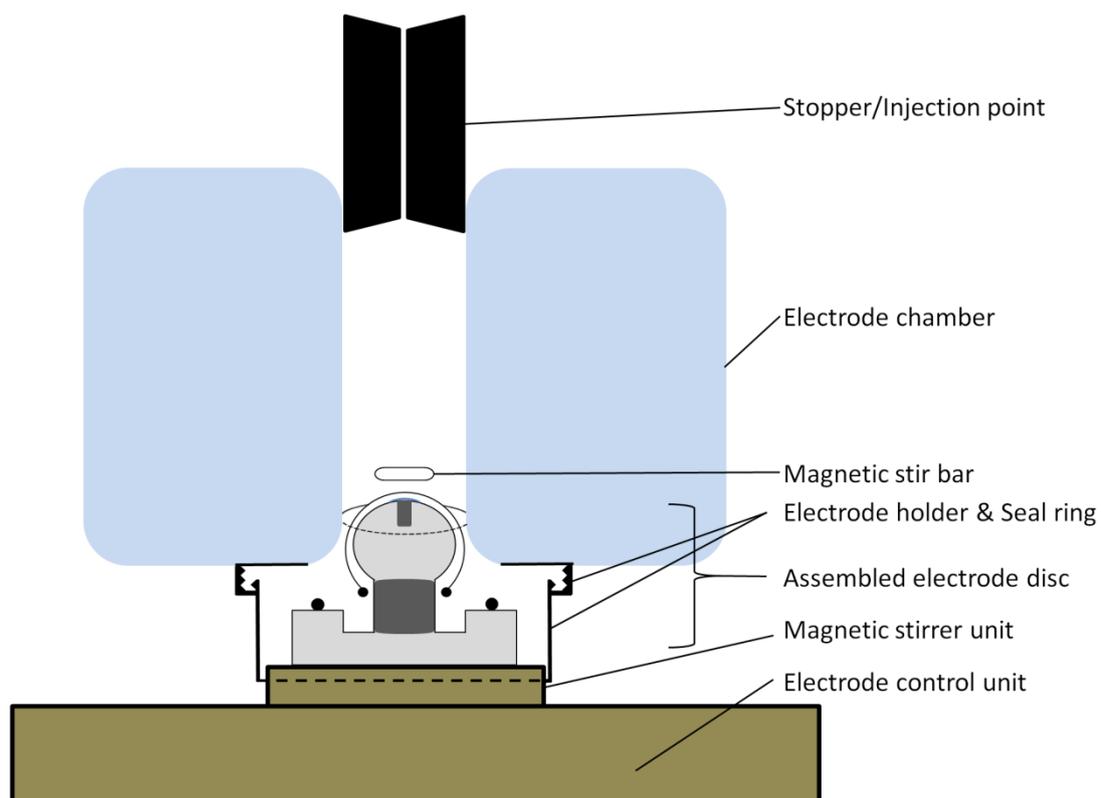


Figure 8 Set up of N₂O Electrode.

The electrode control unit applies a polarising voltage of -1.2 V to the silver-silver electrode disc. The prepared electrode disc sits into an electrode holder and is screwed in the bottom of the electrode chamber. The electrode chamber contains a temperature control unit and has a capacity of 3 mL volume. A stopper ensures minimal gas exchange with the atmosphere and is also used as injection point. A magnetic stir bar is used to mix the solution in the electrode chamber.

2.12.2 Cell preparation

A 500 mL conical flask, containing 500 mL of minimal media, was inoculated with 1% of minimal media overnight culture, sealed with parafilm and grown overnight at 37°C (30°C for *Paracoccus*) statically. The remaining oxygen in the flask was consumed very quickly and the cultures became anaerobic. This was important for the organism to undergo nitrate respiration and to maximally express the enzymes involved in this process, including a N₂O reductase if present. Afterwards, care was taken so that the cells were not exposed to oxygen at any time. Therefore, the cells were split into 10 x 50 mL falcon tubes inside an anaerobic glove box. One millilitre was transferred into a plastic cuvette to determine the OD₆₀₀ of the culture by spectrophotometry (section 2.9). The tubes were covered with parafilm to make them air tight and centrifuged at 4000 x g for 15 min. Back in the glove box, the supernatant was removed and the cell pellets were combined in two falcon tubes. The cells were washed each with 25 µL of nitrate free minimal media (Appendix A), the tubes were sealed with parafilm and the centrifugation step was repeated. Nitrate free minimal media was used to avoid N₂O production from nitrate respiration processes that would interfere with the N₂O injected in the electrode. The supernatant was removed in the glove box and the cell pellet was resuspended in 200 µL nitrate free media. The cells were transferred into a plastic cuvette and parafilm sealed. The cell preparation steps in the anaerobic glove box were performed in between the electrode calibration steps to ensure rapid injection once the electrode was ready for the addition of cells.

2.12.3 Electrode procedure

After the electrode had been set up as described in section 2.12.1, it was connected to the O₂view data acquisition and system configuration software. The electrode response was constantly monitored. Afterwards the chamber was filled with 2 mL of nitrate free minimal media (Appendix A), the stirrer was set to a value of 70, the temperature was set to 37°C (30°C for *Paracoccus*) and the system was left to equilibrate over night for 16-18 hours. Initial tests were performed using 2 mL of 20 mM phosphate buffer (pH 7.6) instead of the nitrate

free media to ensure that the media did not cause an electrode response itself. Successful equilibration was tested by stopping the stirrer briefly, which resulted in a dip in the graph. Next the system was calibrated, which involved two steps: an air line (100% oxygen) and a zero oxygen line. The later was achieved by applying an O₂ scrubbing system consisting of glucose (16 mM), glucose oxidase (4 units/mL) and catalase (20 units/mL) final concentration (Field et al., 2008). A concentrated stock of each solution was prepared (1.6 M, 800 units and 4000 units, respectively). This way only µL amounts (20 µL, 10 µL and 10 µL, respectively) of each solution had to be added to the phosphate buffer in order to achieve the final concentrations. By this method the system remained anaerobic for the whole day. The electrode was set up and calibrated each day. Once calibrated and anaerobic, 2 x 300 µL of a saturated N₂O solution of 19.33 ± 2.78 mM, prepared as described in section 2.12.4, were injected using a 0.5 mL Hamilton syringe (Microlitre™ Syringe). This resulted in an increase in the signal. Some time (2-10 min) was allowed to pass to achieve a fairly stable background rate for the injected N₂O, as a slow gas exchange with the atmosphere occurs due to the open injection point. This rate was later subtracted from the N₂O reduction rate when cells were added. Afterwards 200 µL of washed and concentrated cells (section 2.12.2) were injected and the N₂O response was monitored. A drop in the signal corresponds to the reduction of N₂O to N₂.

2.12.4 Preparation of an aqueous saturated N₂O solution

A 100% saturated N₂O solution was prepared in dH₂O at room temperature (20°C) as follows. A Hungate tube containing 10 mL dH₂O was sparged with pure N₂O (BOC) for 30 min and left over night to equilibrate. A head space concentration of 27.74 ± 2.78 mM was determined via GC analysis as described in section 2.11. The GC method was changed to 95 split instead of the usual 20 split to be able to measure such high N₂O concentrations. A Henry's law constant of 0.697 at 20°C [293.15 K] was used to determine the N₂O concentration of the liquid phase, which was 19.33 ± 2.78 mM. As the N₂O standards (STG), used for calibration, had a maximum of 10,000 ppm, but 100% N₂O have presumably a concentration of 1 mio ppm, additional standards

were prepared manually. A 3 mL Exetainer was sparged with pure N₂O (1 mio ppm) for 3 min at atm pressure. From this, a 1:10 dilution was prepared to design a 100 K ppm standard. The dilution was prepared by removing 300 µL of air from the Exetainer (using a 250 µL gas tight Hamilton syringe; Model 1725 SL SYR) and replacing it with 300 µL of the 1 mio ppm standard. The 100 K ppm standard was then used to prepare a 10,000 ppm standard in the same way. In order to ensure accurate quantifications, only standards that have not been used for dilutions were measured. Thus, several standards of the same concentration were prepared; some of which were solely used for dilutions. The measurements were conducted in duplicate. The GC value of the manually designed 10,000 ppm standard was compared to that of the STG 10,000 ppm standard and showed a variation of only 1%. Thus, the manually designed standards were accurately prepared.

2.13 Drymass constant

The drymass constant is used to convert optical density into drymass. This was needed to compare different biomasses of chemostat experiments and to express their dimensionless quotients (Table 10). One percent of a LB overnight culture was used to inoculate 100 mL of LB. The culture was grown at 37°C shaking and 11 mL of sample were periodically withdrawn. Out of the 11 mL sample 1 mL was transferred into a cuvette and the OD₆₀₀ was determined as described in section 2.9. The remaining sample was transferred into a pre-weighed 15 mL falcon tube and centrifuged at 4000 x g for 10 min. The supernatant was discarded and the pellet was allowed to dry for two days at room temperature. Afterwards the falcon tube, containing the dried pellet, was weighted and the weight of the empty tube was deduced to calculate the dry weight of the pellet. The drymass was divided by 10 to express the drymass in [mg/mL]. The OD₆₀₀ of samples taken at different time intervals was plotted against the respectively measured drymass and the resulting formula was used to convert OD into drymass:

$$\text{Drymass constant} \times \text{OD}_{600} \text{ value} = \text{Drymass [mg/mL]}$$

The drymass constant was 0.5021 and 0.5074 for *Salmonella* and *E. coli*, respectively.

2.14 HPLC

Nitrate and nitrite were determined via HPLC analysis using an anion exchange column Ion Pac AS22, 2 x 250 mm (Dionex ICS-900) as described by the manufacturer. Calibration standards of NO_3^- and NO_2^- were prepared by using different concentrated mixtures of sodium nitrate (NaNO_3^-) and sodium nitrite (NaNO_2^-) (0.25 mM - 2mM). A 1:10 dilution of the chemostat liquid sample, using analytical grade water, was passed through a 0.2 μm Whatman Millipore filter before analysis. The eluent consists of a 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate solution. 10 mM sulphuric acid (H_2SO_4) was used as regenerant. The retention time for nitrate and nitrite was 6.8 minutes and 5.2 minutes, respectively.

2.15 Knock-out mutant construct via the λ -red system

The λ -red system was used, as described in (Datsenko and Wanner, 2000), to create knock-out mutants by replacing the gene of interest with an antibiotic resistance cassette. A PCR fragment (FRT-flanked resistance gene construct) was generated that contains the antibiotic resistance cassette, flanked with 40 bp primers on each site that possessed a nucleotide sequence homologous to one of the gene of interest (Figure 9A). The PCR fragment was designed as described in sections 2.15.1 and purified as described in section 2.3.2, before being electroporated in the strains of interest, expressing the λ -red recombinase of the plasmid pKD46, as described in section 2.15.2 (Figure 9B). Plasmid pKD46 encodes the λ -red bacteriophage recombinase, which is under the control of an arabinose inducible promoter and possesses ampicillin resistance, allowing replacement of the gene of interest with an antibiotic resistance cassette by homologous recombination. The transformed cells were tested for

correct mutation via PCR as described in section 2.15.3 (Figure 9C). Phage P22 transduction was used to remove the plasmid pKD46 to avoid secondary mutations or gene rearrangements and to chromosomally integrate the DNA into a fresh WT background, as described in section 2.15.4. Mutant verification was done by a combined approach of using selective antibiotic plates and PCR.

Removal of the antibiotic resistance cassette was performed by introducing the plasmid pCP20 by electroporation, as described in section 2.15.5. The plasmid contains a FLP-recombinase to remove the antibiotic resistance cassette between the two FRT sites (Figure 9D), as well as an ampicillin resistance gene and is temperature sensitive. This procedure was needed for the creation of a multiple gene knock-out mutant, where the original gene deletion strains possess the same antibiotic resistance cassette, such as the *ΔnsrRΔhcpΔhmpA* triple mutant (Chapter 6).

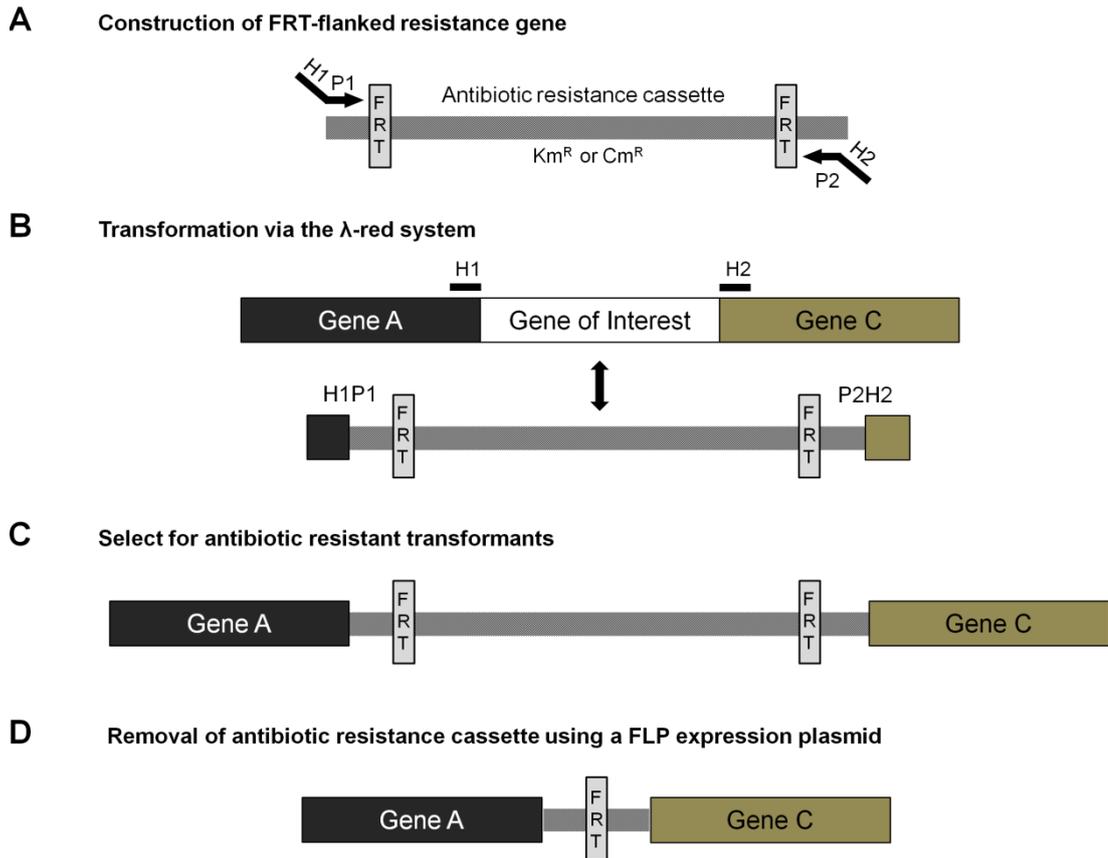


Figure 9 Modified from (Datsenko and Wanner, 2000). Knock-out mutation via the λ -red system

Panel A: Primers (H1P1/H2P2) are designed to be specific to the gene of interest (H1/H2) and the antibiotic resistance cassette of the pKD3 and pKD4 plasmids (P1/P2). These primers are used to amplify the FRT-flanked resistance gene construct.

Panel B: The λ -red bacteriophage recombinase of the plasmid pKD46 replaces the gene of interest with the antibiotic resistance cassette by homologous recombination.

Panel C: The transformed cells are tested for correct mutation via PCR and compared to the wild-type strain. Phage P22 transduction is used to remove the plasmid pKD46 to avoid further gene rearrangements.

Panel D: The FLP recombinase of the plasmid pCP20 recognizes the FRT sites and removes the antibiotic resistance cassette by excision.

2.15.1 Generation of FRT-flanked resistance gene construct

Primers were designed to be specific to the gene of interest (H1/H2) and the antibiotic resistance cassette from the pKD3 (Chloramphenicol resistance, Cm^R) or pKD4 (Kanamycin resistance, Km^R) plasmids (P1/P2) (Figure 9A). H1 and H2 were 40 bp proximal to the coding region of the gene of interest. These were combined with the pKD plasmid primers (P1/P2) at the 3' end of each primer (Figure 9A and Table 4). These primers (H1P1/H2P2) were used to PCR-amplify the FRT-flanked resistance gene construct as described in section 2.3. The PCR product was then purified as described in section 2.3.2.

Table 4 List of primers used for the construction of knockout mutants

Plasmid/ Strain	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
pKD3 and pKD4 (P1/P2)		GTGTAGGCTGGAGCTGCTTC	CATATGAATATCCTCCTTAG
<i>Salmonella</i> SL1344 WT	<i>cueO</i>	ATGTTACGCCGTGATTTCTTA AAATATTCAGTGGCGCTGG	TCAGACCGTAAATCCTAACA TCATTCCCGTATCTTCATGT
<i>E. coli</i> MG1655	<i>cueO</i>	ATGCAACGTCGTGATTTCTTA AAATATTCGTCGCGCTGG	TTATACCGTAAACCCTAACA TCATCCCGTATCTTCATGC

2.15.2 Electroporation

2.15.2.1 Overview

Electroporation was used to introduce plasmid DNA or a linear PCR product (FRT-flanked resistance gene construct, in case of λ -red knockout mutation) into bacterial cells. Electroporation consists of two steps; the production of electro-competent cells and the induction of DNA into these cells, using a short but high voltage electrical pulse. The wild-type strain SL1344, containing the temperature sensitive plasmid pKD46 was transformed with a linear PCR product, designed as described in section 2.15.1. In order to prepare a knockout mutation in the *E. coli* WT strain or *Salmonella* Δ *nsrR* mutant strain, the plasmid pKD46 first had to be extracted (as described in section 2.4) from the pKD46 encoding SL1344 WT strain and electroporated in the respective strain before a PCR product could be introduced.

2.15.2.2 Preparation of electro-competent cells

The recipient strain, containing the plasmid pKD46, was streaked out onto LB_{amp} (containing ampicillin) agar plates and allowed to grow overnight at 30°C. From this a 5 mL LB overnight culture, supplemented with ampicillin, was grown at 30°C under shaking. The overnight culture was diluted 1:100 in 2 x 50 mL of Lennox broth, supplemented with 1 mM arabinose and Ampicillin. Lennox broth was used to maximise colony recovery. The cells were incubated at 30°C with shaking until an OD₆₀₀ of 0.6 was reached after approximately four hours. The starting volume is dependent on the volume of competent cells needed – they are concentrated 100-fold (i.e. 100 ml produces 1 ml cells).

The cells were transferred into 50 ml pre-cooled falcon tubes and centrifuged at 4000 rpm and 4°C for 15 minutes. The cells have to be maintained on ice or 4°C from this point onwards to maximise transformation efficiency. The supernatant was discarded and the pellet was resuspended and washed three times in 25 ml ice-cold 10% glycerol. After the final wash and centrifugation step, the cells were resuspended in 1 mL of ice-cold 10% glycerol. The cells were split into 100 µL aliquots in pre-cooled 1.5 ml Eppendorf tubes and stored on ice till used for electroporation. Left over cells were stored at -80°C. Although electro-competent cells should be produced freshly to ensure maximal transformation efficiency, it was possible to use electro-competent cells from the -80°C storage.

2.15.2.3 Electroporation procedure

Electroporation cuvettes (BIO-RAD) were pre-cooled at -20°C until immediately before use. 50 µL of competent cells were mixed with 10 µL PCR product and placed at the bottom of the electroporation cuvette. The cuvette was placed in the bolt and given a single pulse on EC2 setting on the BIO-RAD MicroPulser at a voltage of 2.5 kV for 5 ms. One mL LB was immediately added, mixed with the cells, then transferred back to the original tube and incubated at 37°C for one hour. During this time the cells recover and recombination occurs. 100 µL of this mix was plated onto LB agar, containing the appropriate antibiotic, to select for transformed cells. The remaining mixture was kept at room

temperature in the case that colonies do not grow and it can be concentrated and plated out.

2.15.3 Knock-out mutant verification

Knock-out mutants were verified by colony PCR, as described in section 2.3.1, to ensure that the correct gene was replaced with the specific antibiotic resistance cassette. The PCR products of a mutant strain were analyzed on an agarose gel and compared to that of the WT strain.

Internal and external verification primers of 20 bp length were designed for each knock-out mutant (Table 5). External primers were designed to be 50-200 bp up-/downstream of the gene of interest, amplifying the antibiotic resistance cassette or the gene of interest in the knock-out mutant or WT strain, respectively. Internal primers amplify a specific nucleotide sequence of the gene of interest in the WT strain, whereas nothing is amplified in the knock-out mutant that has the gene of interest replaced with the antibiotic resistance cassette.

Table 5 List of verification primers for knockout mutant

External primers

Strain	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Salmonella</i> SL1344	<i>cueO</i>	TTAGCATACTGAGAACGTAG	TATGACCTATGAGGTAAACG
<i>E. coli</i> MG1655	<i>cueO</i>	TTTGCTGAGCGAAAAAGACC	TATTGTGGCTTATGCGCTGC

Internal primers

Strain	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Salmonella</i> SL1344	<i>cueO</i>	TATTCCTGACCTGTTAACGG	ATTTCTTCATCAGCATTTGC
<i>E. coli</i> MG1655	<i>cueO</i>	ATTTGCTCACGACCGATGCC	TATGGCCCATCATCTGGCTG

2.15.4 P22 Transduction

P22 transduction consists of three steps. In the first step, production of lysate, the bacteriophage P22 infects the donor cells and takes up its DNA. In the second step, transduction with lysate, the recipient strain incorporates the DNA of the donor strain. In the third step, stable mutants (phage free) are selected using green plates.

2.15.4.1 Production of lysate

A single colony of the verified knock-out mutant was used to set up an LB overnight culture, containing the appropriate antibiotic. 1% (v/v) of this overnight was then subcultured into 10 mL LB and grown aerobically at 37°C for one hour. 20 µL of bacteriophage P22 lysate was added and the culture was grown for additional six hours. Afterwards, 1mL chloroform was added, mixed carefully and left for a minimum of two hours at 4°C. The mixture was transferred into chloroform resistant falcon tubes and centrifuged for 15 min at 5000 x g (MSE Harrier 18/80). The supernatant was removed using a Pasteur pipette and transferred into a fresh falcon tube without touching the chloroform phase. The resulting lysate was stored at 4°C until further used.

2.15.4.2 Transduction with lysate

Overnight cultures of the recipient strain were prepared as described in section 2.2.2. 10 µL of lysate were added to 100 µL of the recipient strain overnight culture and incubated for 45 min at 37°C. The cells were streaked out onto LB plates, containing the appropriate antibiotic and grown overnight. P22 lysate and cells alone were used as negative control.

2.15.4.3 Select for stable mutants

A few of the transduced colonies were streaked out onto green plates and grown overnight at 37°C. Light-green colonies, indicating non-lysogens, were selected and transferred onto fresh green plates. Afterwards light-green

colonies were grown on respective antibiotic resistance plates. Finally the mutants were verified as described in section 2.15.3.

2.15.5 Removal of an antibiotic resistance cassette

Electrocompetent cells of the strain of interest, containing the antibiotic resistance cassette that should be removed, were prepared as described in section 2.15.2.2. 100 μ L electrocompetent cells were transformed with 2 μ L of the temperature sensitive plasmid pCP20 (Cherepanov and Wackernagel, 1995) by electroporation as described in section 2.15.2.3 with the exception that the cells were statically incubated for 1 hour at 30°C. During this time, the recombination event took place, where the FLP recombinase of the plasmid cut out the antibiotic resistance cassette at its FRT sites (Figure 9D). The cells were then incubated overnight at 30°C on LB_{Amp} plates. To ensure the removal of the plasmid, a few colonies were used to inoculate 10 mL LB and grown at 37°C for 6-8 hours. 10 μ L and 100 μ L of a 10⁻⁵ dilution of this culture were spread onto LB agar plates and were incubated overnight at 37°C. A few colonies were then patched, using the same toothpick, onto first LB plates, then LB_{amp} plates and last onto LB plates, containing the antibiotic of the removed cassette, in order to select for successful mutants. Successful mutants, which were only able to grow on plain LB plates, were then confirmed by PCR, resulting in an approximately 100 bp band on the agarose gel.

2.16 RNA extraction and qRT-PCR

Quantitative real-time PCR was used for transcriptional analysis of core nitrate respiration enzymes of *S. Typhimurium* and *E. coli* cells from continuous culture experiments.

RNA extraction was performed by following the protocol of the Promega SV40 Total RNA Isolation System kit with the modification of an extra mRNA stabilisation step during cell harvesting (section 2.16.1) (Tedin and Blasi, 1996,

Rowley et al., 2012) and slight modifications during cell lysis as well as an additional turbo-DNase treatment during RNA extraction and purification procedures (section 2.16.2).

2.16.1 Cell harvesting

Four OD units, sampled from continuous chemostat cultures, were transferred into a pre-chilled 50 mL falcon tube and cell growth was stopped immediately by the addition of 1/5 of the culture volume 5% (v/v) phenol pH 4.3 mixed with 95% (v/v) ethanol. The cells were incubated on ice for 30 min before they were centrifuged at 3220 x g for 10 min at 4°C. After removal of the supernatant, the pellet was resuspended in the residual liquid and transferred to 1.5 mL RNase free Eppendorf tubes. A 60 second centrifugation step at 13780 x g removed the remaining liquid and the pellet was stored at -80°C until further use.

2.16.2 RNA extraction and purification

The pellet was resuspended in 100 µL TE buffer containing 100 mg/mL lysozymes and incubated at 37°C for 15 minutes. Afterwards 75 µL lysis reagent was added, mixed by inverting and an additional 350 µL RNA dilution buffer was added before vortexing. The samples were heated at 70°C for 3 min and centrifuged at 13,000 x g. The supernatant was transferred to a sterile, RNase free Eppendorf tube, 200µL ethanol was added and the mix was transferred to a spin column. Following a 30 second centrifugation step at 13,000 x g, the column was washed with 600 µL wash buffer and centrifuged again for 30 seconds. The flow through was discarded after each centrifugation step. DNase mix, containing 5 µL of 90mM MnCl₂, 40 µL DNase core buffer and 5 µL DNase, was added to the column and incubated at room temperature for 30 minutes. After the addition of 200 µL DNase stop reagent and subsequent centrifugation for 30 seconds, two washing steps with 600 µL and 250 µL wash buffer, respectively, were performed. The column was transferred to a sterile, RNase free Eppendorf tube and 60 µL of RNase-free dH₂O was applied to the column matrix. After allowing the column to stand for 1 minute, the sample was centrifuged at 4500 X g for 2 minutes to elude the purified RNA. An additional

DNase treatment was performed according to the manufacturer's instructions (Ambion® TURBO DNA-free™ Kit) to enhance DNA removal.

2.16.3 Assessment of RNA quality and quantity

RNA quality (check for degradation and DNA contamination) was assessed on a 2% agarose gel (section 2.10) and confirmed with a Bioanalyser (Bio-Rad Experion), using an Experion™ RNA StdSens Analysis Kit according to the manufacturer's instructions, if quality was in doubt. The PCR was performed as described in section 2.16.6 and Table 2 and Table 3. As PCR only works on DNA, it is a good method to check for DNA contaminations in RNA samples. If the RNA is free of DNA contamination, no bands are found on the agarose gel apart from the positive control that contains chromosomal DNA. A 2% gel was used because qPCR primers (section 2.16.4), amplifying a 100 bp fragment, were used. RNA samples were run on the same gel to check for degradation. As mentioned in section 2.10, 1 µL of 5 x loading buffer (Bioline) was added to 1 µL of RNA and 4 µL dH₂O for visualisation. Two clear bands, corresponding to 16S and 23S rRNA subunits, indicated good quality DNA. Degraded RNA had a strong smear under each of the bands. The Bio-Rad Experion Bioanalyser works in a similar way as the agarose gel electrophoresis and the quality can be assessed very precisely on a virtual gel. The quantity of RNA was assessed by using a Nanodrop as described in section 2.9.

2.16.4 qRT-PCR primer design

Specific primers, amplifying a product in the range of 95-105 bp with a melting temperature (T_m) between 58-62°C and a GC content 50-60%, were designed using the *Primer3* Input (version 0.4.0) software (Table 6). The Primers were diluted in a ratio of 1:4 with sigma RNA water to get the working correct concentration. *idnT* and *ampD* were used as housekeeping reference genes. The *Salmonella* primers *ampD*, *narG*, *napA*, *hmpA* and *norV* were kindly provided by Dr. Appia-Ayme. The *Salmonella* primers *nirB*, *nrfA* and *narZ* were designed in this study.

Table 6 Real time quantitative PCR primers***E. coli* MC1000/W3110/MG1655**

Gene	Gene function	Nucleotide sequence (5'-3')
<i>idnT</i>	L-idonate and D-gluconate transporter ; housekeeping gene	F: GTGGGTTTTGTCCTGCTGTT R: ACAGAGAGCGCTGCTACCAT
<i>narG</i>	Cytoplasmic nitrate reductase, alpha subunit	F: GGCAATGTCGATGGTTTCTT R: AGGCAAGTCGCAGTACCAGT
<i>napA</i>	Nitrate reductase, periplasmic, large subunit	F: CCGATCCGAACAAGAAAGTC R: TCAGCAGGTGCAGGTTGTAG
<i>napD</i>	Assembly protein for periplasmic nitrate reductase	F: GAAACGCTGATCCAAACCAT R: GGTGTTTCCTCACCTTGCTC
<i>hmpA</i>	Nitric oxide dioxygenase	F: TTAACGCTATTGCCGCCTAC R: ATCTGGAAGCTGGTGTGCTT
<i>nirB</i>	Nitrite reductase, large subunit	F: GTCGCCGTTGACCATATTCT R: CACCAATACCGCCTACGTCT
<i>nrfA</i>	Cytochrome c nitrite reductase	F: CTTTTGCTGTGACCGATGTG R: GGGCTTTTACAACCTCCAGCA

S. Typhimurium

Gene	Gene function	Nucleotide sequence (5'-3')
<i>ampD</i>	N-acetyl-anhydromuranmyl-L-alanine amidase	F: ATGACGAAAAACCGTCCTTG R: GGATCTATCGTTCCGGTGAA
<i>narG</i>	Cytoplasmic nitrate reductase, alpha subunit	F: TGCTGGTGATGCTTGAAGAG R: CCATTCCGGGTTATTTTCCT
<i>narZ</i>	Cytoplasmic nitrate reductase 2, alpha subunit	F: CGCCAATCGTCTGAAATACC R: ATAGATTCCCATGCCAGCAC
<i>napA</i>	nitrate reductase, periplasmic, large subunit	F: GCGGACAACGGTATTGTCTT R: AATCCTGATTTACCGCGTTG
<i>hmpA</i>	Nitric oxide dioxygenase	F: TTAATGCTATCGCGGCCTAC R: AATCTGGAAGCTGGTGTGCT
<i>norV</i>	Nitric oxide reductase, flavin reductase	F: GTCAGCACTACTGCGACGAG R: CTGAACGGCGTCAGGATATT
<i>nirB</i>	Nitrite reductase, large subunit	F: ACCCCGTAAAGCCTATGACC R: GCCGTGCTTCTCGTAAATC
<i>nrfA</i>	Cytochrome c nitrite reductase	F: GATAAAGCGGGACGATTTGA R: TCATCCCACGGGAACCTAAC

Primer3 Input (version 0.4.0) was used to design the primers. *idnT* and *ampD* were used as housekeeping gene. R: reverse complement primer, F: forward primer.

2.16.5 Genomic DNA preparation

Genomic DNA was prepared following the protocol of the Qiagen Genomic DNA Handbook using 100/G QIAGEN Genomic-tips and solutions from the Qiagen Genomic DNA Buffer set. This allowed isolation of up to 100 µg genomic DNA. Genomic DNA was extracted from 2 x 4 mL of a 10 mL LB overnight and used for standardisation during qPCR.

2.16.6 qRT-PCR

The qRT-PCR was performed according to Rowley *et al.* (2012). 2 µg of DNase-treated RNA were reverse transcribed from random hexamers (Invitrogen), using the Superscript IITM reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The real-time PCR quantifications were performed on the total cDNA obtained, using the Bio-Rad CFX96TM instrument and SensiMixTM SYBR No-ROX kit (Bioline). The SYBR Green only fluorescence if dsDNA is present. During the PCR the single stranded cDNA becomes double stranded and the fluorescence can be measured. Specific qRT-PCR primers were designed as described in section 2.16.4. The real-time PCR experiments were performed on three biological replicates with three technical replicates. The qRT-PCR programme was performed as described in Table 7. Dilutions of genomic DNA, prepared as described in section 2.16.5, were used for standardisation. The calculated threshold cycle (C_t) for each gene was normalized to the C_t of the *idnT* and *ampD* housekeeping gene for *E. coli* and *Salmonella* RNA samples, respectively (Rowley *et al.*, 2012). The Bio-Rad CFX Manager software was used to analyze the data.

Table 7 qRT-PCR programme details

Program	Temperature [°C]	Time [sec]
1) Initial denaturation	95°C	10 min
2) Denaturation	95°C	15
3) Annealing	58°C	15
4) Elongation	72°C	10
5) Repeat steps 2-4	39 X	

2.17 Microarray

Microarray analysis is a reliable, although very expensive and labour intensive method that allows simultaneous analysis of gene expression of every gene of the organism's genome. Microarray slides/chips, commonly made of glass, contain many copies of unique single stranded DNA sequences (cDNA microarray) or oligonucleotides (oligonucleotides microarray) permanently attached to it, forming an array of spots that correspond to a single gene (Figure 10A). This method relies on hybridisation between the DNA of the microarray slide and the RNA samples. The RNA to be analysed is reverse transcribed into cDNA and labelled with fluorescent dyes. Two types of experiments exist. Type 1 labels two different RNA samples with different dyes and competitively hybridises these to the slide, whereas type 2 compares the RNA sample with a reference sample. The type 2, used in this study, has the advantage that multiple data sets can easily be compared. During hybridisation, each fluorescently labelled cDNA molecule will bind to the spot containing its complementary DNA sequence. The hybridised slide is scanned, creating colourful data arrays corresponding to the excited fluorescent dyes of specific genes (Figure 10B).

In this study, 10 µg of RNA samples were labelled with the fluorescent dye Cy5-dCTP (Amersham) and mixed with 1/5 (v/v) of 2 µg Cy3-dCTP dye labelled genomic *Salmonella* SL1344 reference DNA, using the Gibco Bioprime DNA labelling system. The dyed samples were hybridized to an Agilent custom made 8 x 15K oligonucleotides arrays slide and scanned using a GenePix 4000A scanner (Axon Instruments, Inc.). The scan was filtered and quantified using the Genepix Pro 7.0 software (Axon Instruments, Inc.), the data were then normalised using the Batch Anti Banana Algorithm in R (BABAR) (Alston et al., 2010) before they were analyzed using the Gene Spring 7.3 (Agilent) software. Procedures were performed according to (Appia-Ayme et al., 2011) and protocols from the IFR website (<http://www.ifr.ac.uk/safety/microarrays/>). Array slide information can be found on the following website: (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL11416>).

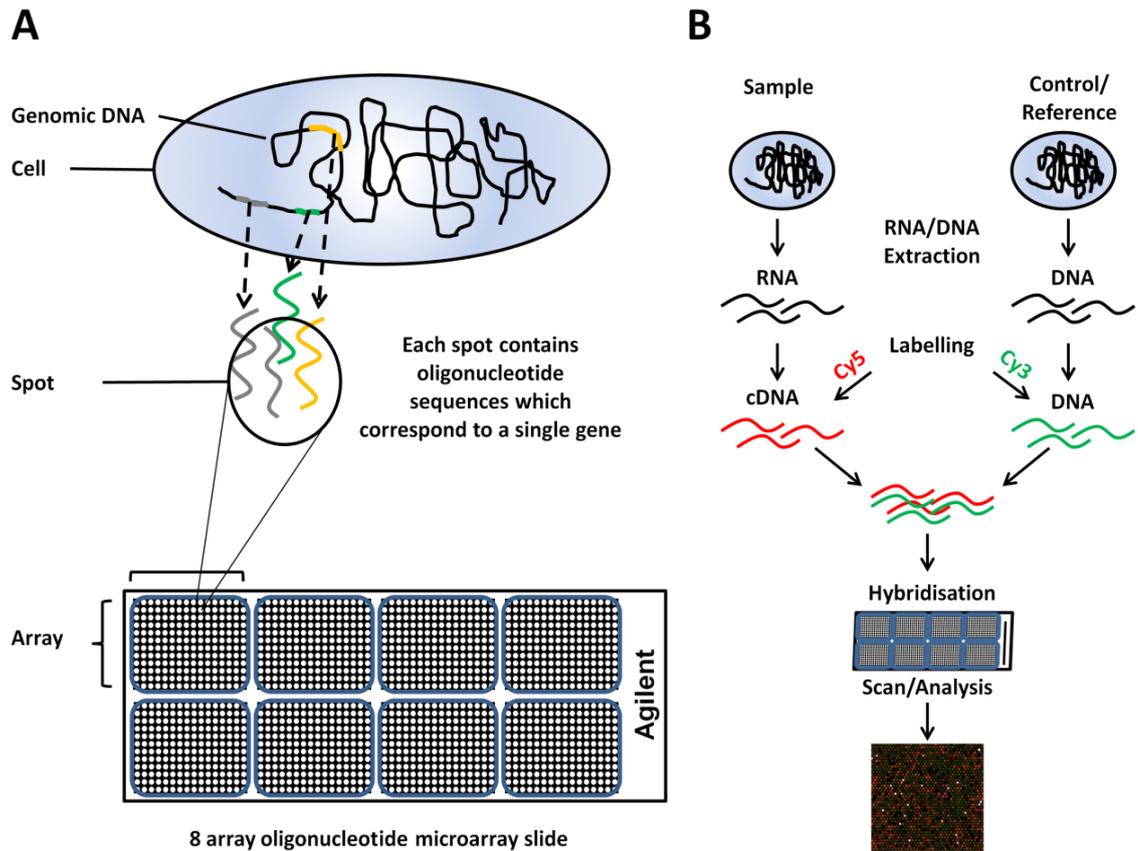


Figure 10 Schematic microarray slide (A) and procedure (B).

(A) A microarray chip/slide contains many copies of unique single stranded DNA sequences (cDNA microarray) or oligonucleotides (oligonucleotides microarray) permanently attached to it, forming an array of spots that correspond to a single gene. (B) A microarray allows simultaneous analysis of gene expression of every gene of the organism's genome. Sample RNA and, in this case, reference DNA (RNA of a different organism or from a different condition could also be used) are extracted. The RNA is reverse transcribed into cDNA and both, sample and reference DNA are labelled with fluorescence dyes (here: Cy5/Cy3, red/green fluorescence). The CyDye labelled DNA mix is hybridised onto the microarray slide, scanned and analysed.

2.17.1 Direct labelling of sample RNA

All reagents and tubes were kept on ice throughout the labelling process. When working with the dye, everything was kept away from light as much as possible. First, random priming reactions were set up in 1.5 mL RNase free Eppendorf tubes by adding 10 µg (minimum concentration = 1300 ng/µL) of RNA and 5 µg of random hexamers (1.66 µL of 3ug/µL stock) in a total volume of 9.4 µL, making up the difference with sigma water. This was incubated at 70°C for 5 min, then chilled on ice for 10 min and vortexed on quick spin. During this time the direct labelling of reference DNA was started. Next 4.6 µL of a reverse transcription reaction master mix, containing 2 µL of 10 x RT buffer (Affinity

Script labelling kit), 2 μL of 0.1 M DTT and 0.6 μL of 50 x dNTP's (prepared by mixing 25 mM dATP, dGTP, dTTP and 10 mM dCTP from Amersham 100 mM stock dNTP) per reaction, was added to the random primer mix. Afterwards, 2 μL of 1 mM Cy5-dCTP (Amersham) fluorescence dye (shows up red on the array) and 4 μL of reverse transcriptase (Affinity Script, StrataGene) were added to achieve a total volume of 20 μL . The mix was incubated at 25°C for 10 min and incubated over night at 42°C in a water bath. The following day 15 μL of 0.1 M NaOH were added and the reverse transcribed RNA (cDNA) was hydrolysed at 70°C for 10 min. To neutralize the solution 15 μL of 0.1 M HCL were added and cooled down to room temperature. Afterwards, clean up with a Qiaquick PCR purification kit (Qiagen) was done as described in section 2.3.2 to remove unincorporated Cy dyes. Twice 50 μL sigma water was used for elution.

2.17.2 Direct labelling of reference DNA

Similar to the RNA labelling all reagents and tubes were kept on ice throughout the labelling process and protected from light when dyes were used. 2 μg of genomic DNA, isolated using the Qiagen Genomic DNA Kit as described in section 2.16.5, were brought to a volume of 21 μL with sigma water. Next, 20 μL of 2.5 x random primer/reaction buffer mix (Gibco Bioprime DNA labelling system) were added. The mixture was boiled for 5 min and then put on ice for 5 min. On ice 5 μL of 10 x dNTP mix (1.2 mM each of dATP, dGTP, dTTP and 0.6 mM dCTP; 10 mM Tris pH 8.0; 1 mM EDTA) were added. Afterwards, 3 μL of 1 mM Cy3-dCTP (Amersham) fluorescence dye (shows up green on the array) and 1 μL of Klenow enzyme (Present in the Kit) were added. This mixture was prepared twice as a eight array slide was used and the mix is only sufficient for five hybridisation reactions. The mixtures were vortexed on quick spin and incubated over night at 37°C. The next day the mixture was cleaned up with a Qiaquick PCR purification kit (Qiagen), as described in section 2.3.2, to remove unincorporated cy dyes. Twice 50 μL sigma water was used for elution. The labelled genomic DNA was mixed 1/5 (v/v) with the labelled cDNA (section 2.17.1).

For hybridisation procedures, the CyDye labelled DNA mix was concentrated in a speed vacuum concentrator (Eppendorf Concentrator 5301) to a final volume of 25 μ L. Adjustment to the volume were made by using sigma water.

2.17.3 Hybridisation

Hybridisation was carried out in a 90 μ L volume per array, 8 x array microarray slide. Powder free gloves were worn at all times when handling the microarrays. The CyDye labelled DNA mix was denatured at 94°C for 2 minutes and cooled down at room temperature for 1 min. 75 μ L of hybridisation buffer (Table 8) was mixed with the 25 μ L of CyDye labelled DNA mix (hybridisation mix). Next an Agilent SureHyb GASKET slide was placed into an Agilent hybridisation chamber, dusted with compressed air and 90 μ L of each sample (hybridisation mix) was aliquoted carefully onto the arrays, without touching the edges of the GASKET. An OGT array slide was placed onto the GASKET with the array side (Agilent label) down and in contact with the hybridisation mix. The clamps were assembled and tightened, ensuring that the bubbles formed were able to move. The assembled microarray slide was hybridised at 55°C for 60 hours in a light tight hybridisation oven while rotating horizontally. The slide was placed in a bath, containing wash solution 1 (Appendix B), and the OGT microarray slide was gently removed from the GASKET slide. The slide was transferred into another chamber, containing wash solution 1, and placed there for 5 min. Afterwards, the same washing procedure was done in chambers filled with wash solution 2 (Appendix B). Before the slide was placed in the scanner, it was dried in a microarray centrifuge.

Table 8 Preparation of hybridisation buffer for 8 x array microarray slide.

Components	Volume [μL] for 90 μL hybridisation	Volume [μL] for one slide with 8 arrays
12 x MES	10	80
5 M Sodium chloride	24	192
Formamide	24	192
0.5 M EDTA	5	40
10% Triton X100	12	96

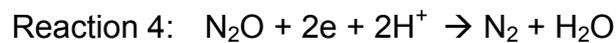
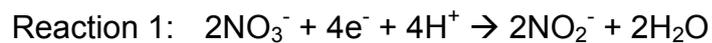
2.17.4 Scanning and analysis

The microarray slide was inserted into the Genepix 4000A (Axon Instruments, Inc.) scanner (Agilent label facing down) and scanned according to the manufacturer's instructions. Note that the scanner produces a mirrored image of the slide on the screen. The scan was filtered and quantified using the Genepix Pro 7.0 software (Axon Instruments, Inc.). This ensured that all spots were aligned and corrected for their signal intensity by subtracting the background signal. Furthermore, the Cy5/Cy3 (red/green) ratio was calculated for each spot. The data were then normalised using the Batch Anti Banana Algorithm in R (BABAR) (Alston et al., 2010). This was important to prevent false interpretation due to differential labelling efficiency. The normalized data were analyzed with the Gene Spring 7.3 (Agilent) software, having a statistically significant minimum cut-off threshold of 2-fold change.

3 Differences in *narG* expression levels contribute to variations in nitrous oxide emissions between *Salmonella* Typhimurium and laboratory strains of *Escherichia coli*

3.1 Introduction

Bacterial denitrification, an important process of the nitrogen cycle, has been extensively studied and is well characterised in soil bacteria; structurally as well as biochemically. Four enzymes carry out the stepwise reduction (Reaction 1-4) of nitrate (NO_3^-) to dinitrogen (N_2) via nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) (Felgate et al., 2012).



Improved understanding of denitrification has important implications in industrial (e.g. waste water treatment plant) and agricultural processes (reducing greenhouse gas emissions) (Takaya et al., 2003, Sullivan et al., 2013).

As mentioned in Chapter 1, enteric bacteria are also able to use NO_3^- for respiratory processes when oxygen is limited; an essential characteristic that allows them to successfully adapt to a range of different natural (soil), commercial (sewage, food processing) and host (the gastro intestinal tract of humans and other warm-blooded animals) environments. Unlike soil bacteria, which produce the potent greenhouse gas N_2O as an intermediate product during denitrification, enteric bacteria undergo only a truncated form of denitrification, resulting in N_2O as their end product (Reaction 3) (Richardson et al., 2009, Arkenberg et al., 2011, Thomson et al., 2012). Lacking the final reduction of N_2O to dinitrogen only makes a minor difference to the bacterium bio-energetically (Richardson et al., 2009). Alternatively, they can reduce NO_3^- to NH_4^+ via the DNRA pathway (Figure 3 and Figure 4). Which of the two pathways will be used depends on the nitrate and carbon availability. It has been shown that soil bacteria, such as *Citrobacter* sp., *Bacillus* sp. or *Paracoccus denitrificans*, as well as enteric bacteria including *Salmonella* and *E. coli* undergo denitrification during a high nitrate to carbon ratio (nitrate-

sufficient/carbon-limited conditions), whereas nitrate ammonification occurs at a low nitrate to carbon ratio (nitrate-limited/carbon-sufficient conditions) (Rowley et al., 2012, Streminska et al., 2012).

The first step of nitrate respiration, the reduction of nitrate to nitrite, is the same process in both denitrification and DNRA. It is accomplished by either the periplasmic Nap, encoded by the *napFDAGHBC* operon or by the integral isoenzymes NarA and NarZ, which are encoded by the *narGHJI* and *narZYWV* operons, respectively and which have their active site located in the cytoplasm (Stewart et al., 2002, Jepson et al., 2007, Rowley et al., 2012, Potter et al., 2001). In the DNRA pathway, the nitrite is then further reduced to ammonium in a second reaction ($\text{NO}_2^- + 6e^- + 8\text{H}^+ \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O}$), performed by either the membrane associated cytochrome c nitrite reductase NrfA in the periplasm or by the cytoplasmic NADH dependent NirB (Wang and Gunsalus, 2000). Thus, Nar and NirB together represent the cytoplasmic pathway for nitrate respiration while Nap and NrfA execute the same process in the periplasm. Activation of each of these pathways depends on the availability of nitrate and nitrite and the carbon to nitrate ratio (Rowley et al., 2012, Streminska et al., 2012).

During denitrification, the cytotoxin nitric oxide (NO) is produced as a side product (Reaction 2) and requires a sophisticated detoxification machinery for the repair of caused damage and cell survival. In addition to the NO produced by their own metabolism, enteric bacteria have to face NO stress from the host immune system (Runkel et al., 2013). *Salmonella* and *E. coli* employ three major enzymes to provide sufficient protection against NO. These include a soluble monomeric flavohaemoglobin (HmpA) which detoxifies two molecules of NO to one molecule of N₂O under anaerobic conditions, a di-iron centred flavorubredoxin, NorV with its NADH-dependent flavorubredoxin oxidoreductase NorW (NorVW) and the cytochrome c nitrite reductase NrfA (Mills et al., 2008, Crawford and Goldberg, 1998, Gilberthorpe and Poole, 2008, Gardner et al., 2002, van Wonderen et al., 2008). NorVW and NrfA detoxify NO to N₂O and ammonia, respectively and function under anaerobic or oxygen depleted conditions. Their importance and expression varies with changing physiological conditions (Mills et al., 2008). Each enzyme is tightly regulated by at least one

of the regulators known to mediate a response to NO, namely NorR (exclusive NO response regulator), NsrR (NO-sensing repressor), FNR (Fumarate and Nitrate Regulator, global switch between aerobic and anaerobic growth) and Fur (Ferric Uptake Regulator). A list of *Salmonella*'s and *E. coli*'s genes, important during anaerobic nitrate respiration and NO detoxification, alongside their regulators is given in Table 9.

N₂O emissions by denitrifying soil bacteria are well documented, especially for the model organism *P. denitrificans* (Felgate et al., 2012, Thomson et al., 2012). In contrast, N₂O production in enteric bacteria has been neglected. Although there have been a few reports on N₂O production of *E. coli*, *Klebsiella pneumonia* and *Salmonella*, little is known about the molecular mechanisms that underpin the differences in release of N₂O by enteric bacteria (Smith, 1983, Bleakley and Tiedje, 1982, Rowley et al., 2012, Satoh et al., 1983).

Studies by Rowley *et al.* (2012) showed that the pathogenic *Salmonella* Typhimurium is able to convert up to 20% of the nitrate catabolized to N₂O (mM levels) when grown anaerobically under nitrate-sufficient conditions and that this is dependent on an active NarG. Other preliminary data of the same group indicated that an *E. coli* strain produces a comparatively low amount of N₂O when grown under the same conditions. This agrees with *E. coli* data from another study which detected only nanomolar N₂O levels (Streminska et al., 2012). As described in Chapter 1, *E. coli* and *Salmonella* possess identical enzymology and known regulatory systems involved in nitrate respiration and NO detoxification. Having this in mind, the question arises: What is the cause of this difference and what might be the reason from the organism's point of view? This chapter describes the nitrate catabolism of three laboratory *E. coli* strains and compares it with that of the related pathogenic bacteria *Salmonella* Typhimurium. A combined microbial physiological and transcriptional approach is used to address these questions.

Table 9 Modified from Cole (2012). Selected genes and their respective regulons in *E. coli* and *S. Typhimurium*

Gene	Transcriptional Regulation	Main Function	References
<i>nar</i>	NarXL (+), FNR (+)	Nitrate reduction (cytoplasm)	(Constantinidou et al., 2006, Pullan et al., 2007)
<i>nap</i>	NarQP (+), FNR (+), NarXL (-), NsrR (-)	Nitrate reduction (periplasm)	(Rabin and Stewart, 1993, Filenko et al., 2007, Potter et al., 2001, Pullan et al., 2007)
<i>hmpA</i>	MetR (+), FNR (-), Fur (-), NsrR (-)	NO detoxification	(Poole et al., 1996, Filenko et al., 2007, Poole, 2005, Membrillo-Hernandez et al., 1998, Karlinsey et al., 2012, Pullan et al., 2007)
<i>nirB</i>	NarXL (+), NarQP (+), FNR (+)	Nitrite reduction (cytoplasm)	(Potter et al., 2001, Wang and Gunsalus, 2000, Tyson et al., 1993)
<i>nrfA</i>	NsrR (-), FNR (+), NarXL (-), NarQP (+)	Nitrite reduction (periplasm)	(Pullan et al., 2007, Bodenmiller and Spiro, 2006, Filenko et al., 2007, Partridge et al., 2009, Page et al., 1990)
<i>norVW</i>	NorR (+)	NO detoxification	(D'Autréaux et al., 2005, Pullan et al., 2007)
<i>hcp-hcr</i>	FNR (+), NsrR (-), NarXL (+), NarQP (+)	NH ₂ OH/NO responsive, controversial	(Filenko et al., 2005, Chismon et al., 2010, Tucker et al., 2010, Karlinsey et al., 2012, Constantinidou et al., 2006, Wolfe et al., 2002, Filenko et al., 2007)
<i>ytfE</i>	NsrR (-), putative FNR (-) and Fur (-) but no obvious Fnr or Fur binding sites were found	Repair of [Fe-S] clusters	(Efromovich et al., 2008, Karlinsey et al., 2012, Justino et al., 2007, Overton et al., 2008, Pullan et al., 2007, Bodenmiller and Spiro, 2006, Justino et al., 2005b, Justino et al., 2006)
<i>nirC</i>	NarXL (+), NarQP (+), FNR (+)	Nitrite transport	(Pullan et al., 2007, Potter et al., 2001)
<i>narK</i>	NarXL (+), NarQP (+), FNR (+)	Nitrate-Nitrite transport	(Constantinidou et al., 2006, Potter et al., 2001)

The (+) indicates transcriptional activation and the (-) indicates transcriptional repression.

3.2 Aim

The aim of this study was to test the hypothesis that there are differences in endogenous N₂O production levels between the closely related *Salmonella* and *E. coli* during anaerobic nitrate respiration and to identify possible reasons behind this.

3.3 Experimental design

3.3.1 Anaerobic Hungate batch

S. Typhimurium SL1344 and three different laboratory *E. coli* strains (MG1655, MC1000 and W3110) were cultured anaerobically in Hungate tubes for 24 hours as described in section 2.7. The MGN media was supplemented with 22 mM nitrate and 5 mM glycerol to achieve nitrate-sufficient/glycerol-limited growth conditions. For the N₂O analysis of a *S. Typhimurium* SL1344 $\Delta narG$ mutant (section 3.5.4.1), 5% (v/v) overnight culture was used as inoculum instead of the usual 2% as the $\Delta narG$ mutant had a growth defect. The OD_{590nm} has been determined spectrophotometrically as described in section 2.9. N₂O was determined by GC analysis as described in section 2.11. For qRT-PCR analysis of *S. Typhimurium* SL1344 and $\Delta narG$, 2% of a LB overnight was used as inoculum to ensure a sufficient biomass (2 OD units) for RNA extraction. Mid-log RNA samples were taken at 4 h (SL1344 WT) and 6 h ($\Delta narG$) post inoculation. The mid-log time points were determined with the help of a growth curve (Figure 21A), performed in an antecedent experiment. RNA extraction and qRT-PCR was performed as described in section 2.16.

3.3.2 Drymass constant

The drymass constant of *S. Typhimurium* and *E. coli* MG1655 has been determined as described in section 2.13. The drymass constant was used to determine the biomass of continuous cultures, which in turn was used to

calculate the dimensionless nitrogen production and consumption quotients (Table 10).

3.3.3 Continuous chemostat cultures

S. Typhimurium SL1344 wild-type (WT) and three different *E. coli* WT strains (MC1000, MG1655 and W3110) were cultured in continuous chemostats as described in section 2.8. N₂O was determined by GC analysis as described in section 2.11. The MGN media was either supplemented with 22 mM nitrate and 5 mM glycerol to achieve nitrate-sufficient/glycerol-limited growth conditions or with 20 mM glycerol and 5 mM nitrate to achieve nitrate-limited/glycerol-sufficient growth. RNA extraction and qRT-PCR was performed as described in section 2.16. Ammonia was determined as described in section 2.6.

Competition chemostats were inoculated with *S. Typhimurium* SL1344 WT and $\Delta narG$ cultures, ensuring that the inoculum of $\Delta narG$ was proportionately higher than that of the WT strain. The cultures were run as described in section 2.8. Liquid samples were taken periodically and used for OD measurements as well as to determine CFU/ml. CFU [%] was determined by making serial dilutions (WT = 10⁻⁵ -10⁻⁸, $\Delta narG$ = 10⁻⁴ -10⁻⁶) on LB and LB_{cm} spread plates, whereas $cfu_{WT} [\%] = cfu_{LB} (WT + \Delta narG) - cfu_{LB_{cm}} (\Delta narG)$. The chemostats either started with nitrate-sufficient growth conditions and were switched to nitrate-limited growth after 120 hours post inoculation, or in reverse order, starting with nitrate-limited growth and switching to nitrate-sufficient growth.

3.5 Results

3.5.1 N₂O production of nitrate-sufficient batch cultures

Anaerobic Hungate batch cultures were used as the initial method to get an idea of the strains' growth behaviour and N₂O production during nitrate respiration. It allowed a quick and easy comparison between the tested strains and served as validation for later chemostat experiments.

S. Typhimurium SL1344 and three different laboratory *E. coli* strains (MG1655, MC1000 and W3110) were cultured anaerobically in Hungate tubes for 24 hours (24 h). 5 mM glycerol was used as a carbon source and 22 mM nitrate as an electron acceptor to perform nitrate respiration. All tested strains followed a similar growth pattern and reached the stationary phase after approximately eight hours (Figure 11A). *Salmonella* reached its optical density (OD) maximum at a value of 0.23 ± 0.01 , whereas the OD_{max} of all *E. coli* strains was slightly higher at 0.33 ± 0.02 . The N_2O production at 24 hours post inoculation is expressed as absolute N_2O levels (Figure 11B) and as N_2O per OD unit (Figure 11C). N_2O production levels at 2 and 6 hours post inoculation are not shown here as they were, with an average of two order of magnitude lower compared to the 24 hour time point, too little to make a reliable interpretation. Nevertheless, it showed that initiation of N_2O production in Hungate batch cultures starts after 6 hours. Thus, N_2O measurements were only taken at 24 hours post inoculation, when performed in Hungate tubes, from then on.

Both, differences between *Salmonella* and *E. coli* and within different *E. coli* strains, in relation to N_2O production, are detectable. The difference between SL1344 and the tested *E. coli* strains is significant when looking at the N_2O levels per OD unit (Figure 11C) but there is no significant difference between the three *E. coli* strains.

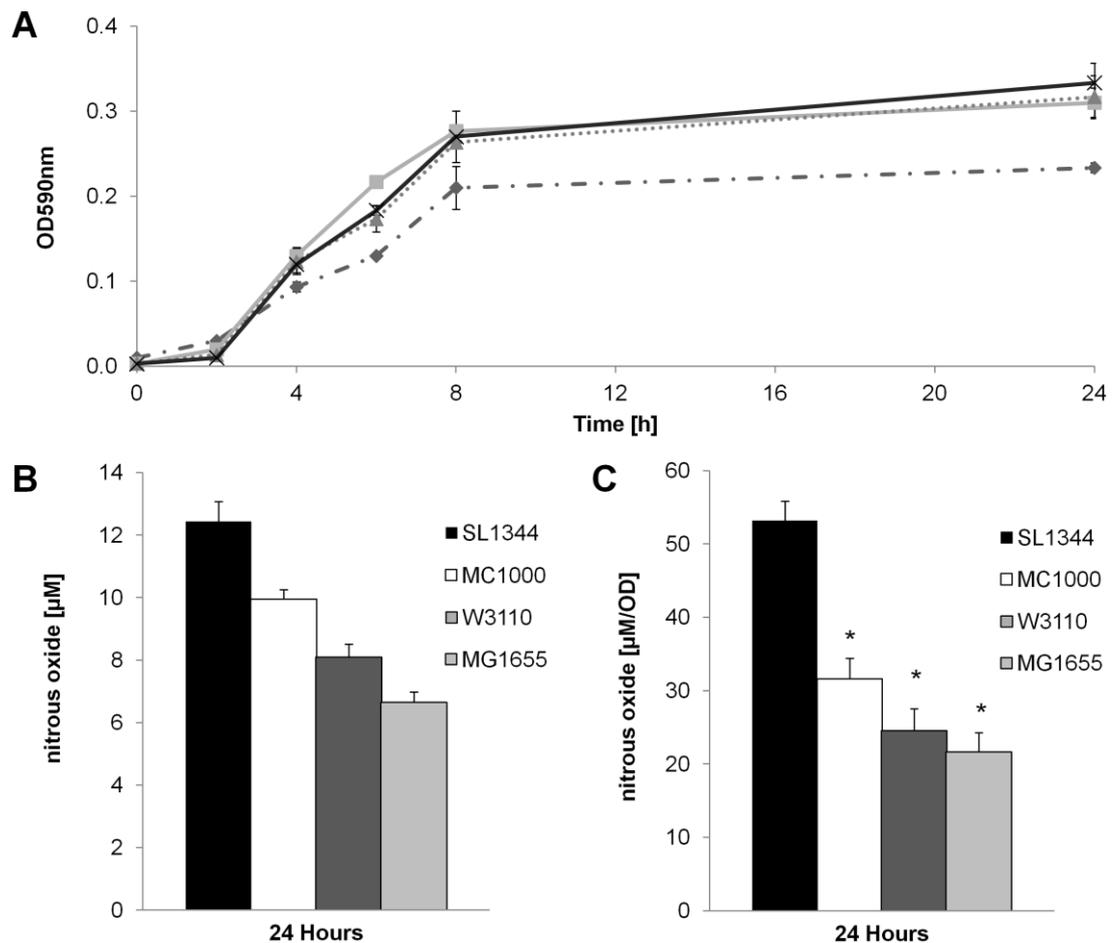


Figure 11 Growth and N₂O production of anaerobic batch cultures during nitrate respiration.

Panel A: Anaerobic growth of *S. Typhimurium* SL1344 (diamond, dashed), *E. coli* MC1000 (triangle, dotted), *E. coli* W3110 (X, dark grey), and *E. coli* MG1655 (square, light grey) in Hungate tubes. OD_{590nm} was taken at 0, 2, 4, 6, 8 and 24 hours post inoculation. The cultures were grown in 10 mL MGN (N⁺/G⁻) medium, inoculated with 2% of a MGN overnight. Data are mean ± standard deviation (n=3). *P* < 0.05 for SL1344 versus all tested *E. coli* strains between t₆₋₂₄. Panels B and C: The nitrous oxide production (absolute levels: B [µM]; relative levels: C [µM/OD unit]) of *S. Typhimurium* SL1344 (black), *E. coli* MC1000 (white), *E. coli* W3110 (dark grey) *E. coli* MG1655 (light grey) was measured after 24 hours post inoculation. Data are mean ± standard error (n=3). **P* < 0.05 for SL1344 versus all tested *E. coli* strains; Panel C.

After 24 hours, *Salmonella* produced approximately 53 μM N_2O per OD unit, accounting for approximately 40% higher N_2O levels compared to *E. coli* MC1000 (31 $\mu\text{M}/\text{OD}$), the highest N_2O producing *E. coli* of all tested strains. *E. coli* W3110 produced approximately 24 μM N_2O and MG1655 produced least with approximately 21 μM N_2O per OD unit.

3.5.2 Nitrate respiration of nitrate-sufficient continuous cultures

The Hungate batch culture experiments confirmed the hypothesis that there are differences in endogenous N_2O production levels between *Salmonella* and *E. coli* during anaerobic nitrate respiration. However, in order to find the potential reason for this, we employed continuous culture so that all parameters could be tightly controlled. The chemostat experiments helped to further understand the strains' behaviour during nitrate respiration and allowed to compare their metabolic profiles. As mentioned in Chapter 2, the steady state is the most important feature of chemostats, as the metabolites can be expressed as dimensionless quotients (Table 10) for direct comparisons between different runs. In order to calculate the dimensionless quotients, which take the biomass into consideration, the optical density had to be converted into drymass.

3.5.2.1 Drymass constant

The drymass constant of *S. Typhimurium* and *E. coli* MG1655 has been determined as described in section 2.13. Figure 12 shows the calibration curves, which were used to determine the drymass constants. The drymass constant was 0.5021 and 0.5074 for *Salmonella* and *E. coli*, respectively. The constant for *E. coli* MG1655 has been used for all *E. coli* strains, as they have the same growth behaviour.

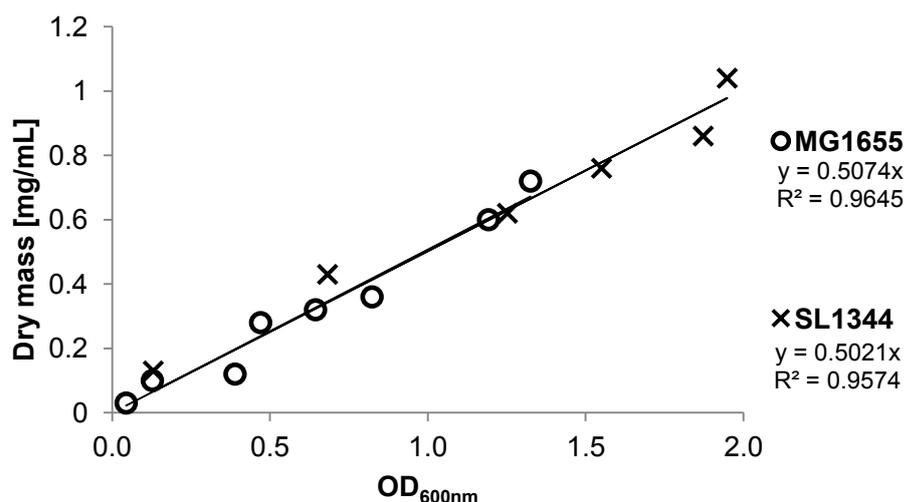


Figure 12 Drymass constant.

Salmonella Typhimurium SL1344 (x) and *Escherichia coli* MG1655 (o) were grown at 37°C shaking. Samples were taken at different time points and the OD value at 600nm was measured. Afterwards the cells were centrifuged and the pellet was dried. The weight of the dried cell pellet was determined and the dry mass constant was determined; Drymass [mg/mL] / OD₆₀₀ value = Drymass constant.

3.5.2.2 Nitrate-sufficient chemostat cultures

Three different *E. coli* WT strains (MC1000, MG1655 and W3110) were cultured in continuous chemostats until an anaerobic steady-state was reached (96-120 h) to try to further understand the differences observed in the batch culture experiments. The continuous cultures were run under glycerol limited (5 mM) and nitrate sufficient (22 mM) conditions according to Rowley *et al.* (2012). All cultures were grown in aerobic batch mode for 22 hours to generate cellular biomass (Figures 13-15A). Afterwards the air supply was switched off and the system was run in continuous mode by attaching feed reservoirs with a dilution rate of 0.05 h⁻¹. The bacteria used up the residual oxygen within 30-60 minutes and the dissolved oxygen remained at 0% throughout the rest of the experiment. Once the system was switched from aerobic respiration to anaerobic nitrate respiration, a decrease in biomass was detectable until a steady state was reached after approximately four bioreactor volume changes (Figures 13-15A). Simultaneously with the switch to the continuous anaerobic mode, the nitrate concentration decreased and nitrite, the product of nitrate reduction, accumulated almost stoichiometrically as a consequence of the respiratory reduction of nitrate to nitrite (Reaction 1) during the first 20 hours of

the transition phase (22-48h) (Figures 13-15B). Taking the *E. coli* strain MC1000 as an example, the nitrate concentration decreased from 23.71 ± 1.47 mM to 2.80 ± 0.35 mM and the nitrite concentration increased from 0 mM to 17.89 ± 2.09 mM. Over the next 50 hours the nitrite concentration decreased by approximately 2 mM to a steady-state value of 15.95 ± 1.64 mM with 1.09 ± 0.50 mM nitrate not being converted. In the steady-state the specific rate of nitrate consumption ($q_{\text{cNO}_3^-}$) was approximately 18% higher than the specific rate of nitrite accumulation ($q_{\text{cNO}_2^-}$) (Table 10). Thus, to account for this difference some of the nitrite must have been further consumed by the culture. As mentioned earlier, nitrite can be further reduced to nitric oxide (Reaction 2). However, NO has not been determined due to its short half life time of 20-30 seconds. Instead N₂O, the end-product of nitrate respiration (Reaction 3), was determined and used to report NO formation. Additionally, increased transcription of *hmpA* (Figures 13-15C), which is regulated by the global NO response regulator NsrR, is another indication for intracellular NO production. N₂O accumulation started as the nitrite concentration decreased in the late transition phase (48-96h, Reaction 3) and reached its maximum of approximately 0.8 mM in the steady-state (Figures 13-15B). For direct comparison between the nitrate and nitrite with N₂O in the steady-state, the N₂O data (Table 10) are expressed as nitrogen-equivalents, as there are two nitrogen atoms in N₂O compared to one in nitrate or nitrite.

In contrast to the MC1000 strain, the specific steady-state rate of nitrate consumption ($q_{\text{cNO}_3^-}$) of MG1655 and W3110 was only ~ 5% and 7% higher than the specific rate of nitrite accumulation ($q_{\text{cNO}_2^-}$) (Table 10). These results support the findings from Hungate batch culture experiments (Figure 11). However, the difference in N₂O levels between the MC1000 strain compared to the other two *E. coli* strains is notably bigger in continuous chemostat cultures. The difference seen in continuous versus batch culture is presumably explainable by the different nature of the two systems. For instance, nutrient limitation in Hungate batch culture is an influencing factor. Furthermore, this study confirmed the preliminary data from a study of the Rowley lab, which indicated a difference in N₂O levels between *Salmonella* and *E. coli*.

E. coli MC1000

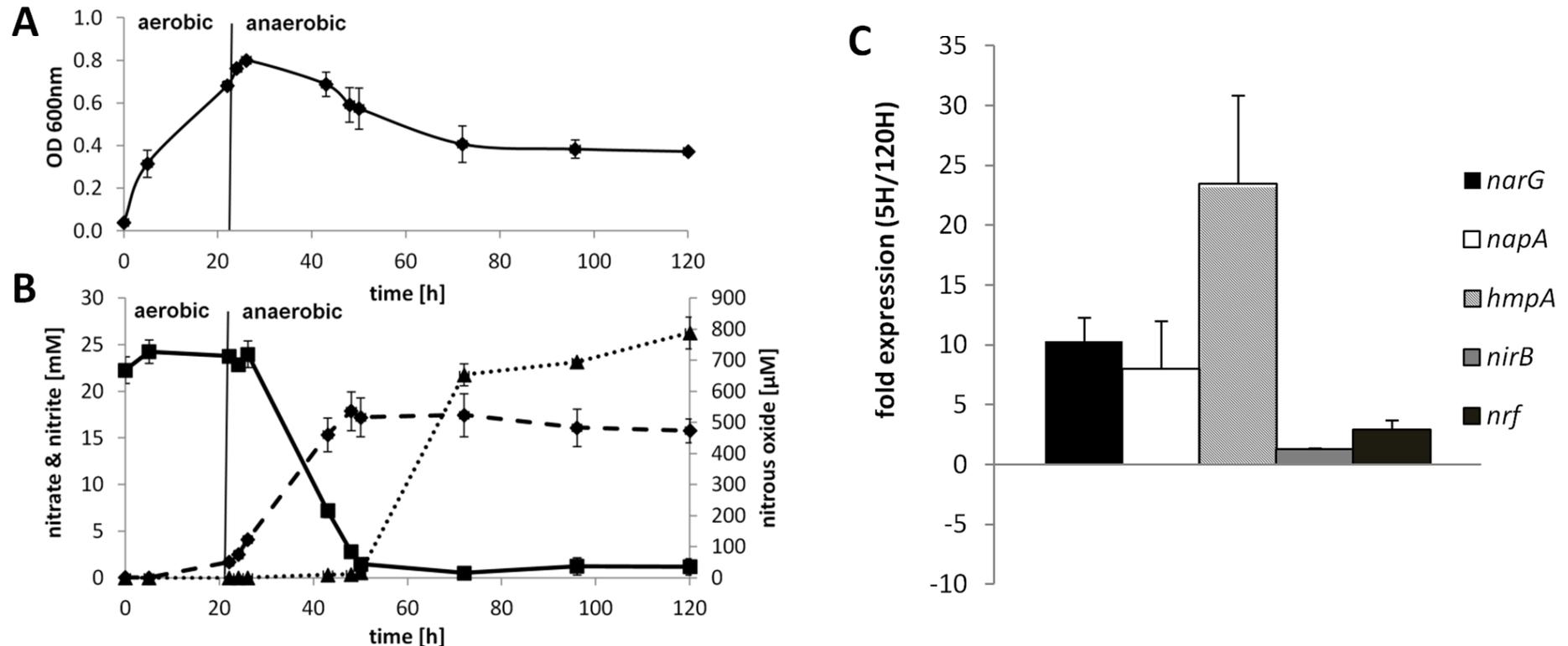


Figure 13 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-limited nitrate-sufficient continuous culture of *E. coli* MC1000.

The culture was initially grown in batch mode under atmospheric oxygen concentration for 22 hours, during which time cellular biomass was generated. The air supply to the culture was then switched off and the system switched to continuous mode at a dilution rate (D) of 0.0467 h^{-1} . The measured DO in the culture fell from 100% to 0% within 1 h after switching off the air supply and was monitored throughout the experiment to ensure it remained at 0%. The pH and temperature were maintained at 7.5 and 37°C , respectively. The glycerol concentration in the feed reservoir was 5 mM and the nitrate concentration was 22 mM to simulate nitrate sufficient growth (Left panels). Nitrate limitation was achieved by using 20 mM glycerol and 5 mM nitrate in the feed reservoir (Right panels) A: Biomass; B: Nitrate (square symbols), Nitrite (diamond symbols) and Nitrous oxide (triangle symbols) C: Gene expression from qRT-PCR analysis - The calculated threshold cycle (C_t) for each gene was normalized to the C_t of the *idnT* control. The values report the fold up/down-regulation at 120 h (anaerobic) relative to the aerobic 5 h time-point. Data are mean \pm standard error ($n=3$).

E. coli MG1655

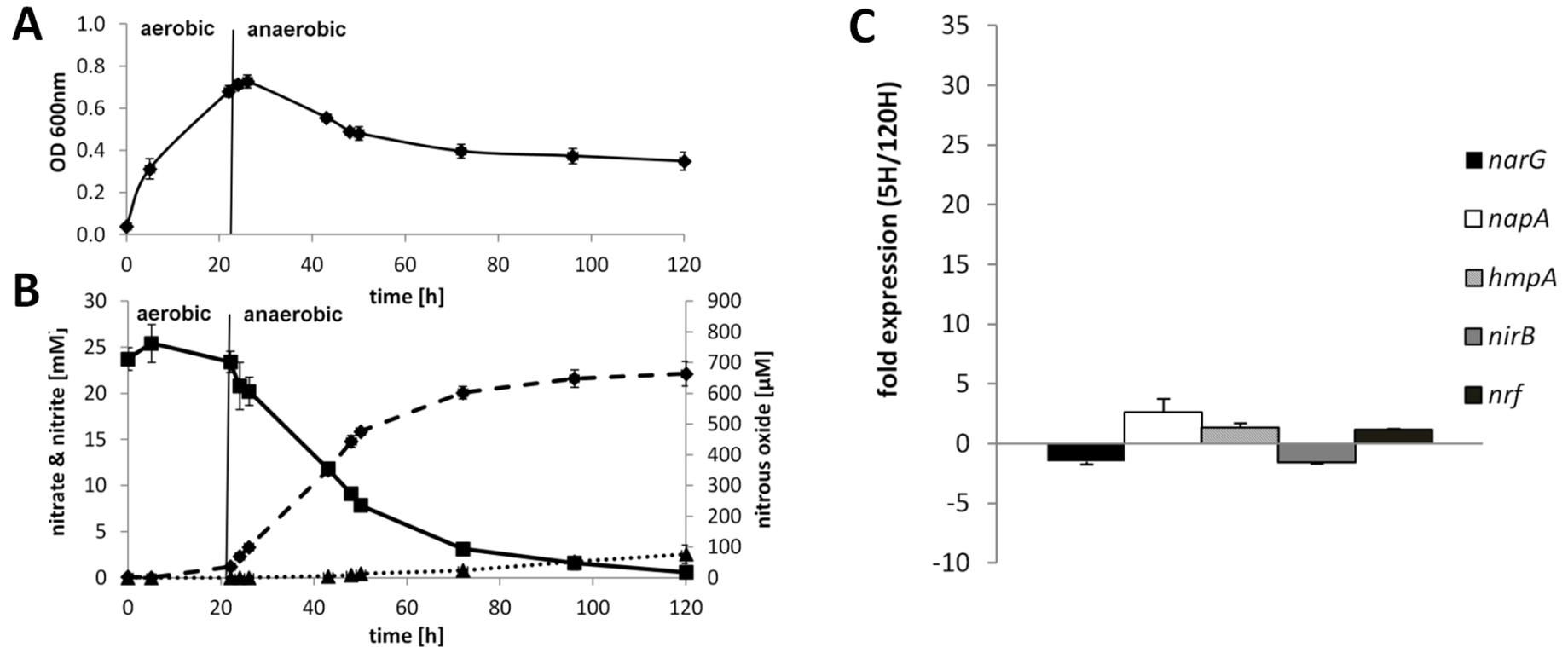


Figure 14 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-limited nitrate-sufficient continuous culture of *E. coli* MG1655.

The culture was initially grown in batch mode under atmospheric oxygen concentration for 22 hours, during which time cellular biomass was generated. The air supply to the culture was then switched off and the system switched to continuous mode at a dilution rate (D) of 0.0467 h^{-1} . The measured DO in the culture fell from 100% to 0% within 1 h after switching off the air supply and was monitored throughout the experiment to ensure it remained at 0%. The pH and temperature were maintained at 7.5 and 37°C , respectively. The glycerol concentration in the feed reservoir was 5 mM and the nitrate concentration was 22 mM to simulate nitrate sufficient growth (Left panels). Nitrate limitation was achieved by using 20 mM glycerol and 5 mM nitrate in the feed reservoir (Right panels) A: Biomass; B: Nitrate (square symbols), Nitrite (diamond symbols) and Nitrous oxide (triangle symbols) C: Gene expression from qRT-PCR analysis - The calculated threshold cycle (C_t) for each gene was normalized to the C_t of the *idnT* control. The values report the fold up/down-regulation at 120 h (anaerobic) relative to the aerobic 5 h time-point. Data are mean \pm standard error ($n=3$).

E. coli W3110

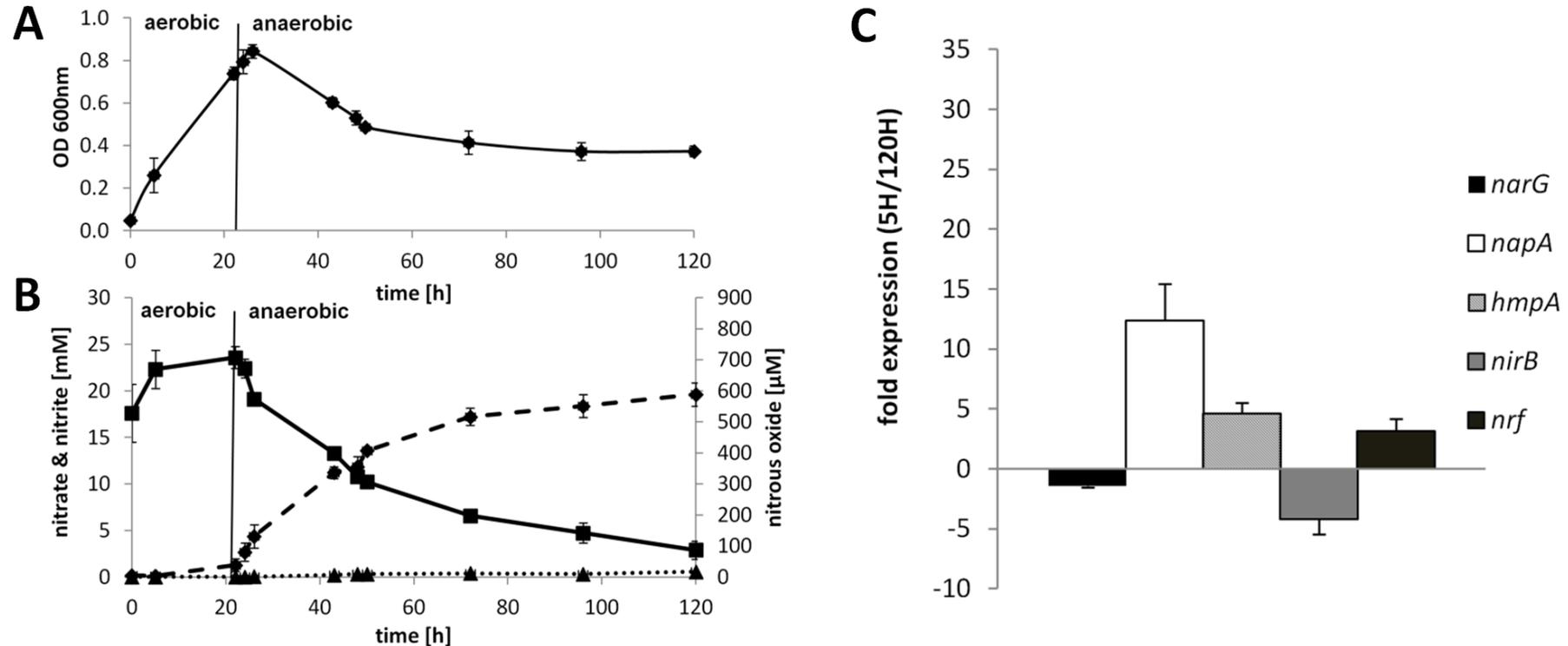


Figure 15 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-limited nitrate-sufficient continuous culture of *E. coli* W3110.

The culture was initially grown in batch mode under atmospheric oxygen concentration for 22 hours, during which time cellular biomass was generated. The air supply to the culture was then switched off and the system switched to continuous mode at a dilution rate (D) of 0.0467 h^{-1} . The measured DO in the culture fell from 100% to 0% within 1 h after switching off the air supply and was monitored throughout the experiment to ensure it remained at 0%. The pH and temperature were maintained at 7.5 and 37°C , respectively. The glycerol concentration in the feed reservoir was 5 mM and the nitrate concentration was 22 mM to simulate nitrate sufficient growth (Left panels). Nitrate limitation was achieved by using 20 mM glycerol and 5 mM nitrate in the feed reservoir (Right panels) A: Biomass; B: Nitrate (square symbols), Nitrite (diamond symbols) and Nitrous oxide (triangle symbols) C: Gene expression from qRT-PCR analysis - The calculated threshold cycle (C_t) for each gene was normalized to the C_t of the *idnT* control. The values report the fold up/down-regulation at 120 h (anaerobic) relative to the aerobic 5 h time-point. Data are mean \pm standard error ($n=3$).

Additionally, the nitrate consumption in the transition phase (22-48 h) of MG1655 and W3110 is slower than that of MC1000 (MC1000 $q_{\text{cNO}_3^-}$: 5.16 vs. MG1655 $q_{\text{cNO}_3^-}$: 3.73 and W3110 $q_{\text{cNO}_3^-}$: 3.00) (Figures 13-15B). Together, this results in a continuous nitrite accumulation in MG1655 and W3110, while MC1000 starts reducing nitrite after approximately 48 hours. This is in turn reflected in the lower N_2O values of MG1655 and W3110 compared to MC1000. MC1000 produced up to 30 times more N_2O , when comparing the N_2O steady state rates of all strains ($q_{\text{pN}_2\text{O}}$) (Table 10).

As mentioned above, ammonium is another possible end-product of nitrate respiration and gets produced by either NirB or NrfA. A study from Rowley *et al.* (2012) showed that *Salmonella* undergoes denitrification during a high nitrate/carbon ratio rather than nitrate ammonification. In contrast to *Salmonella*, which did not produce any ammonia under these conditions, ammonia concentrations in the 0-2 mM range were detectable in the *E. coli* strains (Table 10). This makes sense, as *E. coli* is known to be a natural ammonifying bacterium that produces N_2O only as a side product during nitrate respiration. MC1000 and W3110 produce slightly more ammonia compared to the MG1655 strain, which is also reflected in the higher expression of *napA* and *nrfA* in these strains (Figure 13-15C). The determined ammonium levels must be examined critically as they do not always fit in the nitrogen net balance (nitrogen put into the system; here $[\text{NO}_3^-]$ at t_0 , should equal the sum of all nitrogen equivalents of the steady state ($[\text{NO}_3^-] + [\text{NO}_2^-] + \text{N}-[\text{N}_2\text{O}] + \text{NH}_4^+$ t96-120). Although the nitrogen net balance equals within errors for the nitrate-sufficient growth of all strains, too little ammonia is produced during nitrate limited growth (Table 10). This is due to technical issues with the colourimetric ammonium detection method used in this study. As a result, big variations between triplicate measurements of the same sample were seen. Nonetheless, it was possible to consistently detect higher ammonia levels in nitrate-limited chemostat cultures compared to nitrate-sufficient chemostat cultures (Table 10), reflecting that nitrate ammonification was taking place.

Table 10 Nitrate, nitrite and N₂O steady state rates of chemostat cultures.

Strain	Glycerol at aerobic state [mM]	NO ₃ ⁻	Biomass [g L ⁻¹]	NH ₄ ⁺ produced [mmol L ⁻¹]	NO ₃ ⁻ at steady state	NO ₃ ⁻ consumed [mmol L ⁻¹]	NO ₂ ⁻ produced [mmol L ⁻¹]	N ₂ O produced [mmol L ⁻¹]	qcNO ₃ ⁻ [mmol g ⁻¹ h ⁻¹]	qpNO ₂ ⁻ [mmol g ⁻¹ h ⁻¹]	qcNO ₂ ⁻ [mmol g ⁻¹ h ⁻¹]	qpN ₂ O [mmol g ⁻¹ h ⁻¹]
MC1000	5	23.71 ± 1.47	0.176 ± 0.023	1.80 ± 0.01	1.22 ± 0.88	23.37 ± 1.22	15.95 ± 1.64	1.577 ± 0.102	5.28 ± 0.34	4.30 ± 0.26	0.98	0.383 ± 0.051
		4.14 ± 0.18	0.350 ± 0.028	2.97 ± 0.78	0.09 ± 0.03	4.15 ± 0.50	ND	0.016 ± 0.001	0.52 ± 0.06	ND	0.52	0.002 ± 0.001
MG1655	5	23.59 ± 1.73	0.190 ± 0.019	0.67 ± 0.51	1.09 ± 0.50	22.14 ± 1.83	21.51 ± 1.07	0.106 ± 0.017	5.14 ± 0.39	4.87 ± 0.40	0.27	0.024 ± 0.005
		5.74 ± 0.43	0.335 ± 0.053	2.90 ± 0.78	0.08 ± 0.04	5.18 ± 0.44	ND	0.019 ± 0.002	0.62 ± 0.03	ND	0.62	0.002 ± 0.000
W3110	5	22.90 ± 0.34	0.189 ± 0.021	1.25 ± 0.45	3.82 ± 0.92	19.88 ± 3.07	18.22 ± 1.37	0.019 ± 0.002	4.56 ± 0.68	4.21 ± 0.68	0.35	0.010 ± 0.006
		4.17 ± 0.10	0.389 ± 0.047	2.35 ± 0.19	0.05 ± 0.01	4.10 ± 0.20	ND	0.034 ± 0.032	0.51 ± 0.08	ND	0.51	0.003 ± 0.003

Steady state rates were determined between 96 h and 120 h post inoculation; Aerobic state was determined between 0 and 22 h post inoculation. Nitrate consumption quotient: $qc[NO_3^-] = [NO_3^-]_c * D/X$, where $[NO_3^-]_c$ is the nitrate consumed ($[NO_3^-]_{t0} - [NO_3^-]_{steady\ state}$); Nitrite production quotient: $qp[NO_2^-] = [NO_2^-]_p * D/X$, where $[NO_2^-]_p$ is the nitrite produced ($[NO_2^-]_{steady\ state} - [NO_2^-]_{t0}$); Nitrous oxide production quotient: $qp[N_2O] = [N_2O]_p * D/x$; where $[N_2O]_p$ is the N₂O produced ($[N_2O]_{steady\ state} - [N_2O]_{t0}$); $qc[NO_2^-] = qc[NO_3^-] - qp[NO_2^-]$. Biomass production (qx) = Dx ; where x = the biomass at steady-state ($x = OD * drymass\ constant$; *E. coli*: 0.5074, *S. Typhimurium*: 0.5021) and D is the dilution rate (flow rate of feed reservoir [70 mL h⁻¹] / chemostat volume [1500 mL]) of 0.05 h⁻¹. N₂O data are expressed as nitrogen equivalents ($[N-N_2O]_p = 2 * [N_2O]_p$) to allow comparison with NO₃⁻ and NO₂⁻. Data are mean ± standard error (n=3) apart from *S. Typhimurium* under nitrate sufficiency which was done in duplicate only. ND = not detectable, nitrite detection limit: 0.005 mM.

3.5.2.3 Transcriptional analysis of nitrate-sufficient chemostat cultures

In order to find an explanation for the differences in N₂O levels between *Salmonella* and *E. coli*, and between the three *E. coli* strains, transcriptional analysis of key enzymes involved in nitrate respiration and NO detoxification was performed. Transcriptional *Salmonella* data from Rowley *et al.* (2012) are used for comparisons made between *Salmonella* and *E. coli*. Comparison of the expression levels revealed that the high N₂O levels can be associated with an increased activity of NarG, HmpA (Figures 13-15C) and NorVW (Rowley *et al.*, 2012). This agrees with our previous *Salmonella* study, where we further suggested that N₂O production is linked to nitrite reduction to NO by NarG (Reaction 2) (Rowley *et al.*, 2012). Consequently, when *narG* is knocked out (Rowley *et al.*, 2012) or when it is not active (Figures 13-15C; MG1655 and W3110 strains), N₂O production is significantly decreased. The argument that NarG is involved in the reduction of nitrite to NO is also supported by other studies (Smith, 1983, Metheringham and Cole, 1997, Ji and Hollocher, 1988, Gilberthorpe and Poole, 2008). The produced NO is quickly detoxified to N₂O (Reaction 3), which is reflected by increased transcription of *hmpA* (Figures 13-15C) and *norVW* (Rowley *et al.*, 2012). Although *nirB* has been implicated to generate NO (Weiss, 2006) and the fact that it is up-regulated under nitrate-sufficient conditions in the high N₂O producing strains *E. coli* MC1000 and *Salmonella* SL1344, it has been shown that it does not contribute to an increase in N₂O levels, as a *nirB* mutant showed identical behaviour compared to a wild-type strain (Rowley *et al.*, 2012). The same is true for NrfA.

3.5.3 Nitrate respiration of nitrate-limited continuous cultures

In order to achieve nitrate-limited and glycerol-sufficient growth conditions, 5 mM nitrate and 20 mM glycerol were used. These values were based on results from Rowley *et al.* (2012) that suggested a nitrate concentration of < 17 mM and a glycerol concentration of > 5mM, as a net consumption of 17 mM nitrate and 5 mM glycerol was detectable during nitrate-sufficient growth of *Salmonella*. Furthermore, using the same amounts made comparisons between the two studies easier. The growth pattern (Figures 16-18A) is similar to that of cultures

grown under N^+/G^- conditions (Figures 13-15A) but a higher OD was achieved during the aerobic growth phase; reflecting the greater carbon availability. Although the decline in OD is faster, once the cultures have been switched to the anaerobic continuous mode, the final OD at 120 hours is similar to that of nitrate-sufficient grown cultures. It was noticeable that the nitrate became reduced to nitrite even slightly before the culture was switched to anaerobic growth. This might be a consequence of the high biomass, so that the air pumped through the system was not sufficient for the growth and needed support by nitrate respiration. Nitrate was consumed entirely within a short period of time, consistent with nitrate limitation, and was not detectable in the steady-state. Nitrite levels were detectable in the form of a single peak between two and 43 hours, before it fell to zero levels in the transition phase, from where it stayed zero throughout the steady-state (Figures 16-18A). Nitrous oxide production differed significantly to that of N^+/G^- cultures. Like nitrite, N_2O accumulated transiently with having its peak maximum between 24 and 43 hours post inoculation, before it fell to approximately 5 μM , where it stayed level throughout the rest of the experiment (Figures 16-18B). However, it is notable that the decline in N_2O after its peak maximum is steeper at the beginning and flattens towards the end. This leads to the hypothesis that *Salmonella* and *E. coli* might possess a yet undiscovered nitrous oxide reductase (N_2OR). If this assumption is correct it would be a N_2O reductase with a weak reduction potential, as N_2O declines at a rate of approximately $0.2 \text{ nM mg}^{-1} \text{ min}^{-1}$ (Figure 18B, 26-50 h time point). This hypothesis will be tested and further discussed in Chapter 4. The rate of nitrous oxide production (qp_{N_2O} , (Table 10) was one - and two-order of magnitude lower than for MG1655 and W3110 or MC1000 and SL1344 N^+/G^- cultures. This is reflected in the transcriptional analysis (Figures 16-18C), with Nap being heavily induced rather than Nar and HmpA. Under these conditions the bacteria undergo nitrate ammonification rather than nitrate respiration. These results are consistent with previous studies (Rowley et al., 2012, Potter et al., 1999, Potter et al., 2001, Wang et al., 1999, Stewart et al., 2003, Cole, 1996).

E. coli MC1000

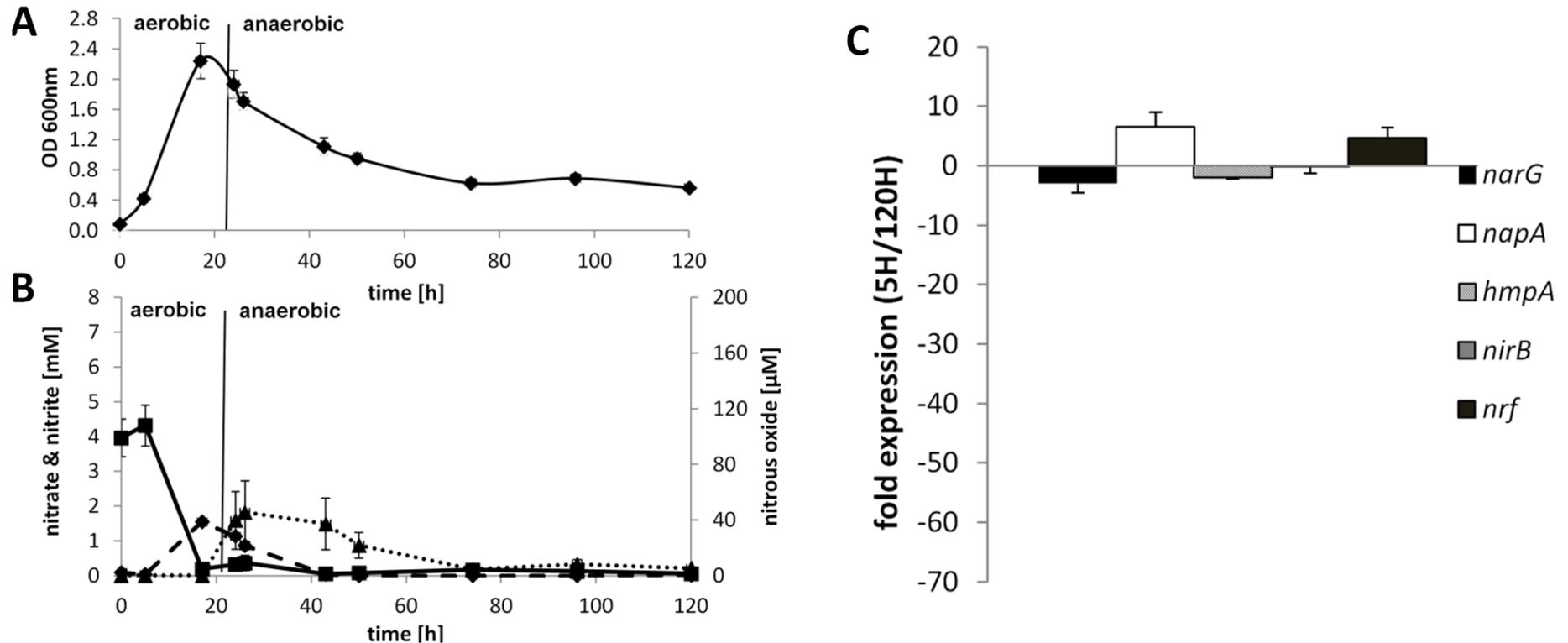


Figure 16 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-sufficient nitrate-limited continuous culture of *E. coli* MC1000.

The culture was run as described in Figures 13-15. The glycerol concentration in the feed reservoir was 20 mM and the nitrate concentration was 5 mM to simulate nitrate-limited growth. A: Biomass; B: nitrate (square symbols), nitrite (diamond symbols) and nitrous oxide (triangle symbols); C: Gene expression levels from qRT-PCR analysis - The calculated threshold cycle (Ct) for each gene was normalized to the Ct of the *idnT* control. The values report the fold up/down-regulation at 120 h (anaerobic) relative to the aerobic 5 h time-point. All data are mean \pm standard error (n=3)

E. coli MG1655

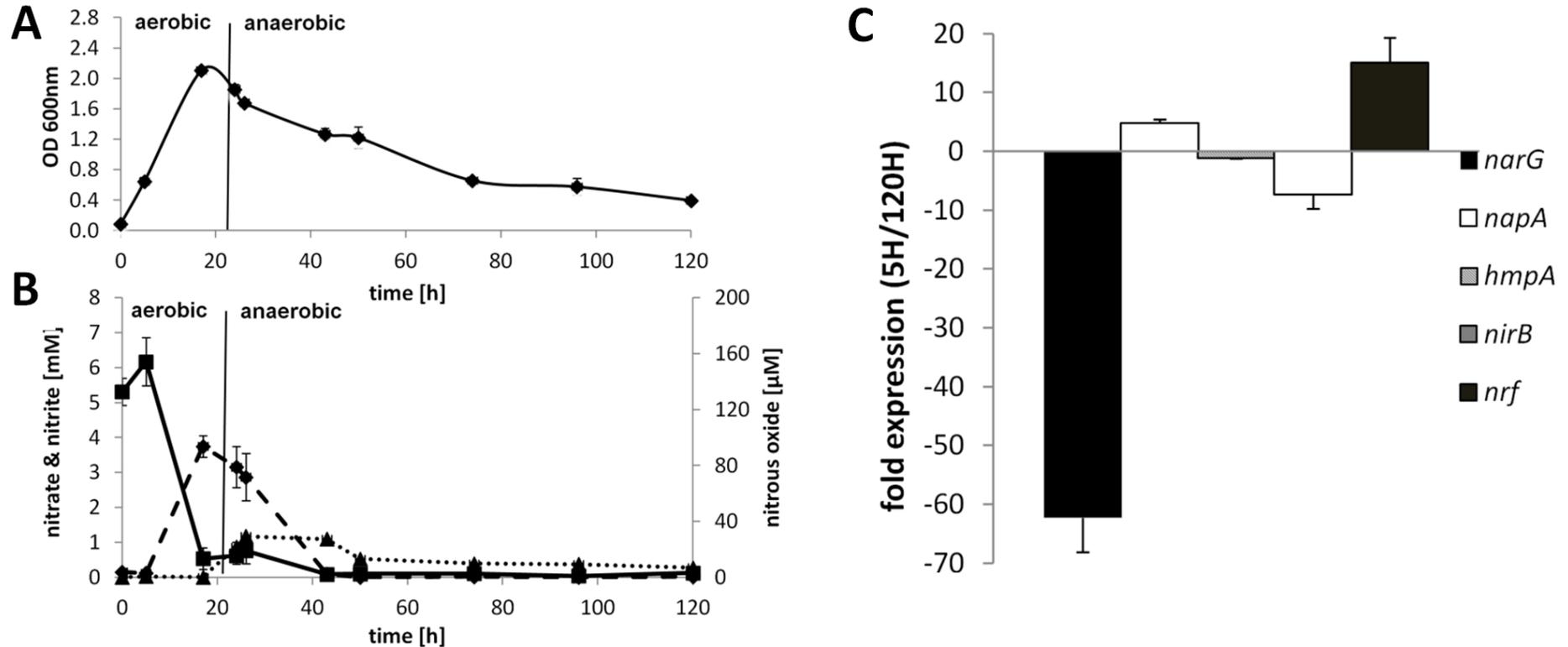


Figure 17 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-sufficient nitrate-limited continuous culture of *E. coli* MG1655.

The culture was run as described in Figures 13-15. The glycerol concentration in the feed reservoir was 20 mM and the nitrate concentration was 5 mM to simulate nitrate-limited growth. A: Biomass; B: nitrate (square symbols), nitrite (diamond symbols) and nitrous oxide (triangle symbols); C: Gene expression levels from qRT-PCR analysis - The calculated threshold cycle (Ct) for each gene was normalized to the Ct of the *idnT* control. The values report the fold up/down-regulation at 120 h (anaerobic) relative to the aerobic 5 h time-point. All data are mean \pm standard error (n=3)

E. coli W3110

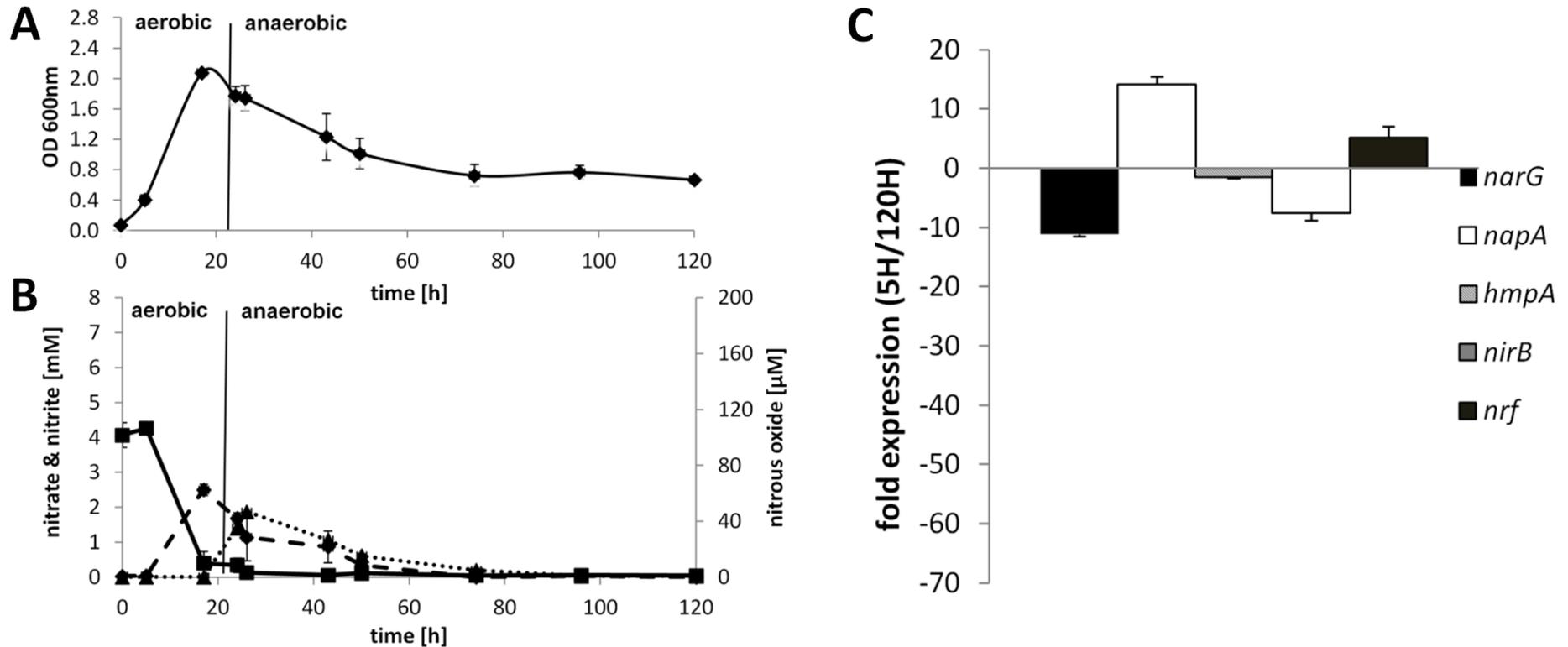


Figure 18 Nitrate consumption, nitrite and nitrous oxide production and gene expression in a glycerol-sufficient nitrate-limited continuous culture of *E. coli* W3110.

The culture was run as described in Figures 13-15. The glycerol concentration in the feed reservoir was 20 mM and the nitrate concentration was 5 mM to simulate nitrate-limited growth. A: Biomass; B: nitrate (square symbols), nitrite (diamond symbols) and nitrous oxide (triangle symbols); C: Gene expression levels from qRT-PCR analysis - The calculated threshold cycle (Ct) for each gene was normalized to the Ct of the *idnT* control. The values report the fold up/down-regulation at 120 h (anaerobic) relative to the aerobic 5 h time-point. All data are mean \pm standard error (n=3)

3.5.3.1 Transcriptional analysis of nitrate-limited chemostat cultures

As mentioned above, NarG is the main enzyme responsible for nitrate reduction during N^+/G^- growth, whereas NapA is the only active nitrate reductase when nitrate is limited. *napA* and *nrfA*, which together represent the periplasmic nitrate respiration pathway for nitrate ammonification, were up-regulated in all tested strains (Figures 16-18C). Furthermore, ammonium was detected as the only product, as there was no accumulation of nitrate or N_2O (Table 10).

Table 11 gives a summary of *narG* and *napA* expression levels, comparing the aerobic (5 h) with the steady state transcription (120 h).

Table 11 Schematic representation of qRT-PCR data of *S. Typhimurium* and *E. coli narG* and *napA* expression levels during anaerobic nitrate respiration.

Organism	Gene	Nitrate Sufficiency	Nitrate Limitation
<i>Salmonella</i> SL1344	<i>narG</i>	↑↑	→
	<i>napA</i>	↑	↑
<i>E. coli</i> MC1000	<i>narG</i>	↑↑	↓
	<i>napA</i>	↑↑	↑↑
<i>E. coli</i> W3110	<i>narG</i>	↓	↓↓
	<i>napA</i>	↑↑	↑↑
<i>E. coli</i> MG1655	<i>narG</i>	↓	↓↓↓
	<i>napA</i>	↑	↑

The qRT-PCR was performed in triplicates, with three independent total RNA preparations taken from nitrate-limited (5mM)/ carbon-sufficient (20mM) chemostat cultures and nitrate-sufficient (22mM)/ carbon-limited (5mM) chemostat cultures. Arrows indicate the up/down-regulation at 120 h (anaerobic) relative to the aerobic 5 h time-point with: no change in expression (→), up/down - regulation (↑/↓; ≤5-fold), strong up/down - regulation (↑↑/↓↓; >5-fold), very strong down regulation (↓↓↓; >50-fold). *Salmonella* data are taken from Rowley *et al.* (2012)

An up-regulation of *narG* expression explains the higher N_2O levels (mM) measured in MC1000 and SL1344 compared to μ M levels seen in MG1655 and W3110, where *narG* stays approximately level or is even slightly down-regulated (Figures 13-15C). Differences in N_2O levels between MC1000 and SL1344 might correlate to a stronger up-regulated *napA* in MC1000, which competes stronger with *narG* for nitrate and thus results in a N_2O production rate, which is approximately half of that of the SL1344 strain (Table 10).

3.5.4 The importance of NarG during anaerobic nitrate respiration.

The experiments above demonstrated the importance of NarG during anaerobic nitrate respiration and showed that there is a direct link between high N₂O production and the expression of *narG*. Thus, to investigate this further, a *Salmonella narG* mutant was compared to the WT strain. Three different experiments were performed to look for variation in the growth pattern (section 3.5.4.1 and 3.5.4.3) and the transcriptional profile (section 3.5.4.2) in both, batch and continuous cultures.

3.5.4.1 N₂O production of nitrate-sufficient batch cultures of *S. Typhimurium* WT and $\Delta narG$

Salmonella WT and $\Delta narG$ were cultured anaerobically in Hungate tubes for 24 hours. Preliminary data showed that the *narG* mutant has a growth defect during anaerobic nitrate respiration. Therefore, 5% (v/v) overnight culture was used as inoculum instead of the usual 2%. This growth defect is not only reflected in a lower OD_{max} ($\Delta narG$: 0.19 vs. WT: 0.24) (Figure 19A), but also results in a slower maximum growth rate ($\mu_{max\Delta narG} = 0.010$ vs. $\mu_{maxWT} = 0.025$). The N₂O production levels are shown in (Figure 19B and C). After 24 hours the *Salmonella* WT strain produced between 60% (N₂O/OD unit) (Figure 19C) and 70% (N₂O absolute) (Figure 19B) more N₂O compared to the *narG* mutant. This result confirms that NarG is essential for N₂O production and that *Salmonella* requires NarG to grow normally in nitrate-sufficient batch cultures. Furthermore, it agrees with $\Delta narG$ chemostat data (Rowley et al., 2012) that showed a 97% reduction in N₂O levels of the *narG* mutant (~ 60 μ M) compared to the WT strain (~ 2.3 mM). In addition, the N₂O levels of the *narG* chemostat mutant (Rowley et al., 2012) compare astonishingly well with the N₂O levels of the *E. coli* MG1655 and W3110 chemostat cultures (Figure 14B and Figure 15B), which had reduced expression of *narG* (Figure 14C and Figure 15C).

3.5.4.2 Competition chemostat of *S. Typhimurium* WT vs. *narG* mutant

Competition chemostats of *S. Typhimurium* WT vs. $\Delta narG$ were performed in order to test the hypothesis that the WT strain should have a selective growth advantage during nitrate-sufficient growth conditions, whereas $\Delta narG$ should have a selective growth advantage during nitrate-limitation. The reasoning behind this is that NarG is known to be the most active enzyme during nitrate-sufficient growth, while primarily Nap is used during nitrate-limited growth. As the *narG* mutant has to rely on Nap for nitrate respiration, it was hypothesized that the Nap in the $\Delta narG$ strain is more active than the Nap of the WT strain, which has a functional NarG in addition, and should therefore result in a growth advantage during nitrate-limitation. Two chemostats were inoculated with WT and $\Delta narG$ cultures, ensuring that the inoculum of $\Delta narG$ was proportionately higher than that of the WT. This was done to compensate for the mutant's growth defect that has been detected during N^+/G^- batch growth. One culture mixture was initially grown under N^+/G^- growth conditions and switched to N^-/G^+ conditions after 120 hours (Condition 1) (Figure 20A), while the other chemostat experiment was performed in reversed order ($N^-/G^+ \rightarrow N^+/G^-$; Condition 2) (Figure 20B). Under both conditions (1 and 2) the WT strain rapidly outgrows the *narG* mutant even before the chemostats were switched to nitrate respiration after 22 hours. When a steady state had been achieved, only 7% of the bacteria were chloramphenicol resistant, representing the *narG* mutant (Condition 1) (Figure 20A). Under condition 2 (Figure 20B), $\Delta narG$ represents only 2.5% of the total biomass between 96 -120 h. By the time the switch from one condition to the other occurs, the biomass of $\Delta narG$ is so little that it has no chance to recover.

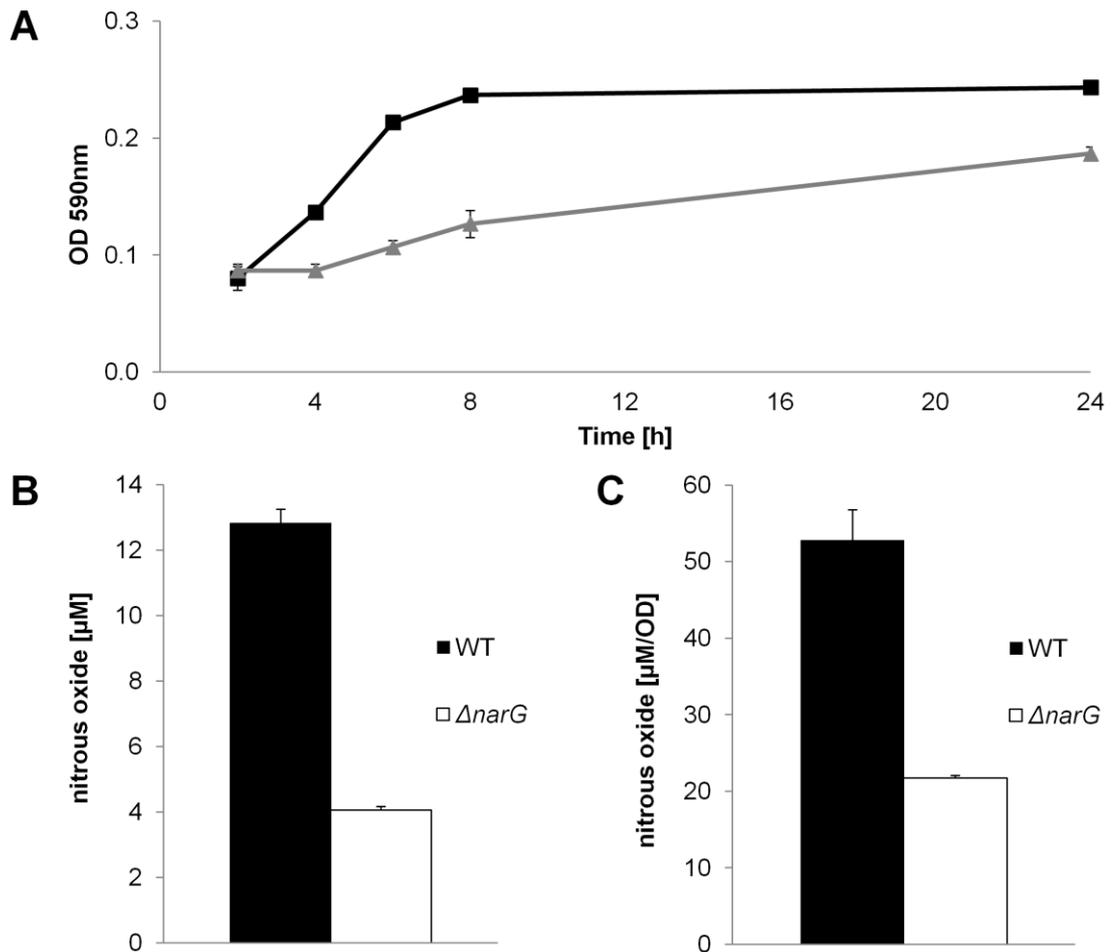


Figure 19 Growth and N₂O production of anaerobic batch cultures of *S. Typhimurium* wild-type and a *narG* mutant during nitrate respiration.

Panel A: Anaerobic growth of *S. Typhimurium* SL1344 WT (black squares) and *S. Typhimurium* SL1344 $\Delta narG$ (grey triangle) in Hungate tubes. OD_{590nm} is taken at 2, 4, 6, 8 and 24 hours post inoculation. The cultures were grown in 10 mL MGN (N⁺/G⁻) medium, inoculated with 5% of a MGN overnight. Data are mean \pm standard deviation (n=3). Panel B and C: The nitrous oxide production (absolute levels: B [μM]; relative levels: C [$\mu\text{M}/\text{OD}$ unit]) of *S. Typhimurium* SL1344 WT (black) and *S. Typhimurium* SL1344 $\Delta narG$ (white) was measured after 24 hours post inoculation. Data are mean \pm standard error (n=3).

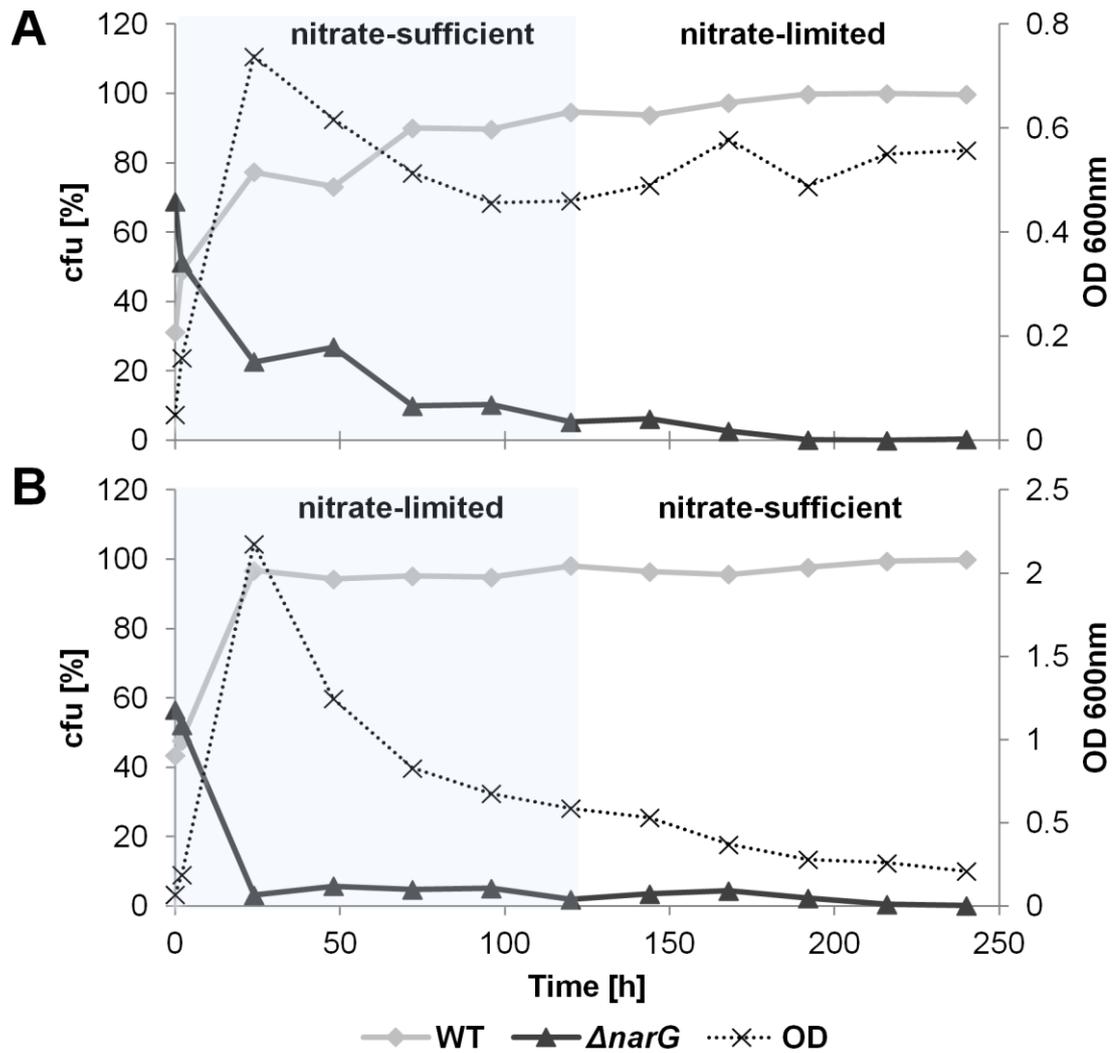


Figure 20 Competition chemostat of *S. Typhimurium* WT vs. $\Delta narG$.

A chemostat was inoculated with WT (diamond, light grey) and $\Delta narG$ (triangle, dark grey) cultures, ensuring that the inoculum of $\Delta narG$ was proportionately higher than that of the WT strain. The cultures were run as described in Figures 13-15. Liquid samples were taken periodically and used for OD measurements (x, dotted line) as well as cfu determination. The cfu [%] was determined by making serial dilutions (WT = 10^{-5} - 10^{-8} , $\Delta narG$ = 10^{-4} - 10^{-6}) on LB and LB_{cm} spread plates; cfu_{WT} [%] = cfu_{LB} (WT + $\Delta narG$) - $cfu_{LB_{cm}}$ ($\Delta narG$). A: The glycerol concentration in the feed reservoir was 5 mM and the nitrate concentration was 22 mM to simulate nitrate-sufficient growth. After 120 hours post inoculation the culture was switched (condition 1 highlighted as light blue background compared to white background for condition 2) to nitrate-limited growth by changing the feed reservoir to 20 mM glycerol and 5 mM nitrate. B: As described for panel A, but this time the culture started with nitrate-limitation and was switched to nitrate-sufficiency after 120 hours post inoculation. Data are from a single experiment.

3.5.4.3 qRT-PCR analysis of nitrate-sufficient batch cultures of *S. Typhimurium* WT and $\Delta narG$

Transcriptional analysis of nitrate-sufficient batch cultures of *Salmonella* WT and $\Delta narG$ was performed in order to determine whether any of the alternative nitrate reductases, NapA or NarZ, can compensate for the loss of NarG activity. The growth curve of *Salmonella* WT and $\Delta narG$ is shown in Figure 21A. In order to reach a higher biomass that is sufficient for RNA extraction, LB overnight cultures instead of MGN overnight cultures were used as inoculum. Apart from the higher biomass, the WT strain reached the stationary growth phase approximately two hours earlier than the *narG* mutant (Figure 21A). This is the result of a faster growth rate. The same is true when comparing the growth pattern of the two *Salmonella* WT strains (LB overnight) (Figure 21A) and (MGN overnight) (Figure 11A). Figure 21C shows that NarZ is, although slightly up-regulated in the *narG* mutant, only poorly expressed in general. This agrees with data from a previous study (Potter et al., 1999). The periplasmic nitrate reductase Nap is activated in both strains Figure 21B. However, to our surprise, *napA* expression is decreased in the *narG* mutant compared to the WT strain. Nonetheless, these data explain the growth defect seen in the *narG* mutant (Figure 19A and Figure 21A) and highlights again the importance of possessing a functional NarG.

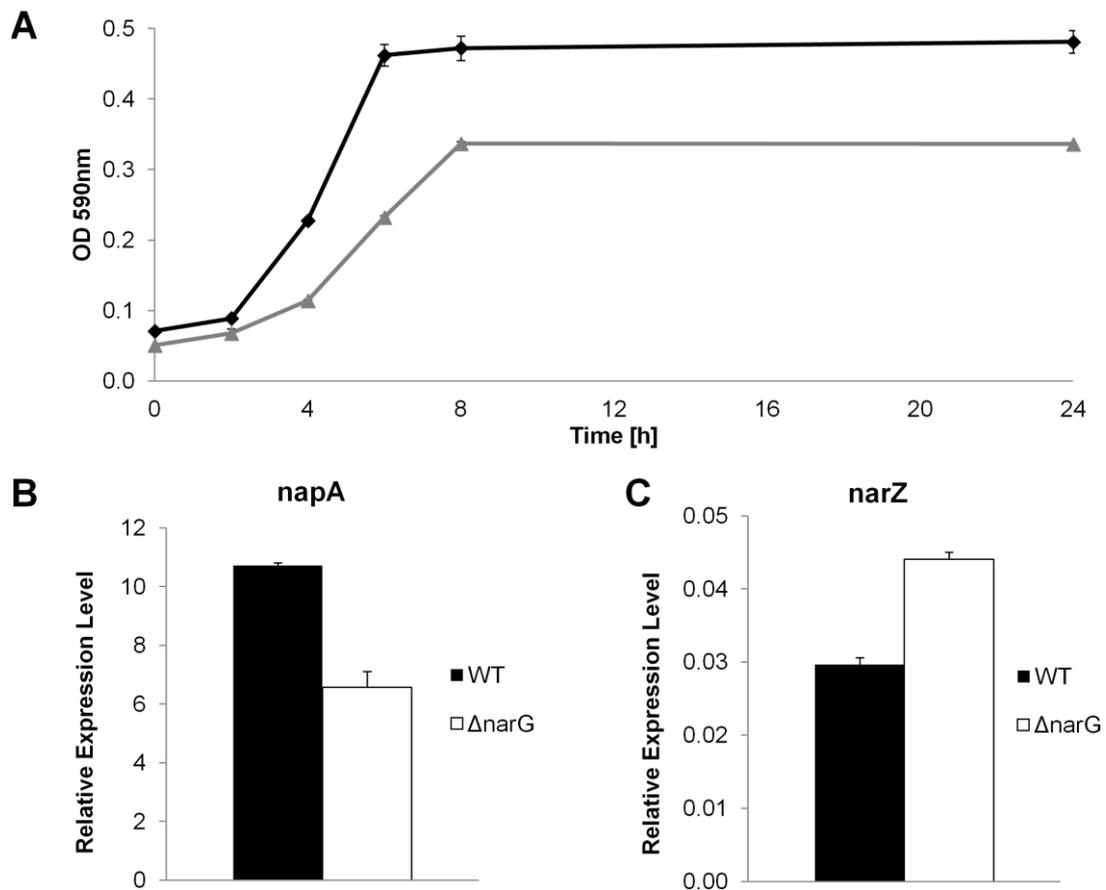


Figure 21 qRT-PCR analysis of anaerobic batch cultures of *S. Typhimurium* wild-type and a *narG* mutant during nitrate respiration.

Panel A: Anaerobic growth of *S. Typhimurium* SL1344 WT (black diamonds) and *S. Typhimurium* SL1344 $\Delta narG$ (grey triangle) in Hungate tubes. OD_{590nm} is taken at 2, 4, 6, 8 and 24 hours post inoculation. The cultures were grown in 10 mL MGN (N⁺/G⁻) medium, inoculated with 2% of a LB overnight. Data are mean \pm standard deviation (n=3). Panel B and C: Gene expression levels of *napA* (B) and *narZ* (C) of SL1344 WT (black) and SL1344 $\Delta narG$ (white) from qRT-PCR analysis. Mid-log RNA samples were taken at 4 h (SL1344 WT) and 6 h ($\Delta narG$) post inoculation. The calculated threshold cycle (Ct) for each gene was normalized to the Ct of the *ampD* control. Data are mean \pm standard error (n=3).

3.6 Discussion

3.6.1 Anaerobic nitrate respiration of Hungate batch cultures

The significance of nitric oxide production is well studied in relation to human or murine macrophages that produce NO as part of the immune response against pathogens. However, in comparison little is known about the endogenous NO production and its subsequent reduction to N₂O by enteric bacteria. N₂O production has been extensively studied in soil bacteria due to its huge global warming potential, but it has been widely neglected in enteric bacteria.

Preliminary data from the Rowley laboratory showed that there are differences in N₂O production levels between the closely related bacteria *Salmonella* and *E. coli* during anaerobic nitrate respiration. The pathogenic *Salmonella* produced larger quantities of this laughing gas, although it possesses, to the best of our knowledge, identical enzymology as *E. coli* to perform these reactions. This study directly continues from these results and aimed to validate the preliminary findings and to find the reasons behind this.

In this chapter, the endogenous N₂O production of *S. Typhimurium* and three *E. coli* strains was measured in anaerobic batch and continuous chemostat culture under nitrate-sufficient/glycerol-limited and nitrite-limited/glycerol-sufficient growth conditions. Since nitric oxide is highly reactive and will quickly become detoxified by the conversion to nitrous oxide in the cytoplasm of *E. coli*, intracellular NO concentrations were quantitatively determined by measuring extracellular N₂O production, the end-product of NO detoxification (Rowley et al., 2012). Furthermore, transcriptional analysis of the core genes known to be involved in anaerobic nitrate respiration was performed.

Anaerobic batch culture experiments, using Hungate tubes, showed that there are significant differences in N₂O production between *Salmonella* and *E. coli* (Figure 11). *Salmonella* produced approximately 40% more N₂O/OD unit

compared to *E. coli* MC1000, the highest N₂O producing *E. coli* strain. Additionally, differences between the three *E. coli* strains were detectable.

These results confirmed the preliminary data and raised the question of what is the cause for this difference? Previous studies and genomic analysis showed that both, *E. coli* and *Salmonella* possess the same NO detoxification mechanisms (Potter et al., 2001, Chaudhuri et al., 2008) (Figure 3 and Figure 4, Table 9). One reason might be differences in the regulation or transcription of genes involved in NO detoxification. Other reasons could be differences in the enzymes' reaction rates or that other, yet unknown enzymes and pathways exist.

3.6.2 Anaerobic nitrate respiration of chemostat cultures

In order to confirm the findings from the Hungate batch culture experiments and to find a possible explanation for the differences in N₂O production, continuous chemostat experiments were performed. The chemostat experiments confirmed the findings from the Hungate batch culture experiments and the differences in N₂O levels between the three *E. coli* strains were even more apparent. *E. coli* MC1000 produced the highest amount of nitrous oxide under nitrate-sufficient culture conditions, converting approximately 7% of the consumed nitrate into N₂O (nitrogen equivalents), whereas the conversion of MG1655 and W3110 accounted for only 0.5% and 0.1%, respectively (Table 10). Beside the fact that *Salmonella* and *E. coli* have the same nitrate respiration and NO detoxification enzymes, N₂O production is very different. In comparison with our previous study, *E. coli* MC1000 behaves more similar to *Salmonella* with respect to N₂O production (Rowley et al., 2012). Nonetheless, the rate of nitrous oxide production (qpN₂O) of MC1000 is only approximately half of that of *Salmonella* (Rowley et al., 2012).

To gain further insight into the responses to NO and to understand the reasons for different N₂O levels of closely related organisms (*E. coli* and *Salmonella*) and between different *E. coli* strains, the transcriptional expression of key enzymes involved in the NO detoxification pathways were determined. This part

of the study revealed that the highest N₂O levels were achieved under nitrate-sufficient growth condition, where the expression of *narG* and *hmpA* was maximal. Furthermore, it demonstrated that *narG* is the key enzyme responsible for differences in N₂O production (Figures 13-15C), which support findings of previous studies (Rowley et al., 2012, Gilberthorpe and Poole, 2008). Despite up-regulation of *nrfA*, nitrite rather than ammonium is the major product of nitrate respiration, when nitrate is abundant (Figures 13-15B and Table 10). A possible explanation can be found when looking at the e⁻ flux of nitrate respiration compared to nitrate ammonification (Rowley et al., 2012, Simon et al., 2008). The 1H⁺/e⁻ stoichiometry for the reduction of nitrite by NrfA is lower than for nitrate reduction by Nar under electron acceptor sufficient conditions (Simon et al., 2008). For the same reason it makes bioenergetic sense (at the biological/cellular level) to use Nar rather than Nap for nitrate reduction, if possible (Rowley et al., 2012).

However, under nitrate-limited conditions, where only small amounts of N₂O are produced (Figures 16-18B), Nap and NrfA are the dominant enzymes (Figures 16-18C). A higher biomass and a rapid utilization of the limited nitrate and nitrite available reflect that *E. coli* undergoes ammonium production via the DNRA pathway rather than nitrate respiration, when nitrate is limited. This is consistent with previous studies and was also confirmed by the ammonia data (Table 10) (Rowley et al., 2012, Potter et al., 1999, Potter et al., 2001, Wang et al., 1999, Stewart et al., 2003, Cole, 1996). Although different expression of *narG* has been identified as a cause for the differences seen in the N₂O production of the tested *E. coli* strains and in comparison to *Salmonella* (Rowley et al., 2012), reasons for the different *narG* expression need further investigation and remain speculative. From a transcriptional point of view, comparing the activity of the two component regulatory systems NarX-NarL and NarQ-NarP would be the most obvious starting point. Differences in the nitrate and nitrite transporter systems NarK and NirC is another plausible explanation. For instance, NirC, which imports nitrite from the periplasm into the cytoplasm, could be impaired in the low N₂O producing strains. As a result most of the nitrite is trapped in the periplasm and therefore does not become converted to NO by the cytoplasmic NarG. As a consequence, less NO is available for HmpA and NorVW to

produce N₂O. Another possibility is differential regulation by the transcriptional regulator FNR, which is the global switch between aerobic and anaerobic growth. It was noticeable that the nitrate reduction rate of MC1000 was higher compared to that of MG1655 and W3110 during the transition phase, which could be a result of differences in the FNR gene. As the genome sequence of MC1000 is unavailable, the wider FNR region (including 200 bp up-/downstream of FNR) of MC1000 and MG1655 was sequenced by the genome analysis centre (TGAC, Norwich). As MG1655 and W3110 have a 100% homology in this region, it was sufficient to sequence only that of MG1655. The result showed that the FNR region of all three *E. coli* strains has a 100% homology at the nucleotide as well as amino acid level.

In order to explain the different N₂O production of *E. coli* MC1000 (Table 10) *Salmonella* (Rowley et al., 2012) during N⁺/G⁻ growth conditions, the transcriptional profiles need to be considered carefully. Although their transcriptional profile is very similar, *napA* expression is higher in *E. coli*. Thus, the competition between Nar and Nap for nitrate is higher in *E. coli*, resulting in lower N₂O production. This makes sense when looking at the ammonium production. *E. coli* produced ammonium as well as N₂O during nitrate-sufficiency, while *Salmonella* did not produce any ammonium and has therefore higher N₂O levels.

Differences between *Salmonella* and *E. coli* can be further explained by comparing their natural habitats and lifestyles. For instance, in nitrate-rich environments, such as fertilized soil or waste water treatment plants, Enterobacteriaceae that possess both Nap and Nar, will benefit from using NarG as opposed to nitrate-limited environments, where the use of NapA will be advantageous. The same is true for the human host environment, where nitrate concentrations vary with the dietary intake (McKnight et al., 1997) or during an infection state, leading to increased gastric NO (Dykhuisen et al., 1996). Due to its pathogenic nature, *Salmonella* is likely to encounter high levels of NO through contact with macrophages (Alam et al., 2008) and therefore evolved very efficient detoxification mechanisms, resulting in high N₂O production. The same mechanisms are used by *Salmonella* and *E. coli* to detoxify their

endogenously produced NO during nitrate respiration. However, the opportunistic *E. coli*, which cannot survive the high NO levels encountered in the intracellular environment, tries to remove the highly toxic NO as quickly as possible and thus uses other pathways that do not result in high N₂O production. This is reflected in differential regulation patterns seen in *E. coli* (Figures 13-15C) compared to *Salmonella* (Rowley et al., 2012). Furthermore, it makes sense for *E. coli* to use nitrite ammonification instead of nitrite reduction to N₂O, as this process is energetically higher (1H⁺/e⁻ of 2 vs. 1.5) (Rowley et al., 2012). Consequently, the question arises as to whether there is a general correlation between pathogenicity and high N₂O production.

3.6.3 The importance of NarG

The experiments discussed above showed that *narG* has the biggest impact on the endogenous N₂O production of *Salmonella* and *E. coli*. Thus, *Salmonella* WT vs. $\Delta narG$ batch (Figure 19 and Figure 21) and chemostat culture (Figure 20) experiments have been performed. Figure 19 shows that the *narG* mutant produced approximately 60% less N₂O/OD unit compared to the WT. It was important here to consider the N₂O production levels in relation to the OD of the cultures, as it was noticeable that the *narG* mutant had a growth defect. This supports the role of NarG as a major energy source for nitrate-sufficient growth conditions and shows that the alternative nitrate reductases NarZ and NapA are unable to compensate for the loss of NarG (Rowley et al., 2012, Potter et al., 1999). Transcriptional analysis supported these results (Figure 21B and C). Competition chemostat experiments (Figure 20) further showed, that the WT culture even has a selected advantage during nitrate-limited growth conditions. In conclusion, this study revealed that there are differences in endogenous N₂O production levels between closely related organisms and even between bacterial sub-strains of the same species. The major reason for this was found to be different expression levels of *narG*.

3.7 Future work

Although it was shown that different expression of *narG* was the major cause for differences seen in N₂O levels among the tested strains, further experiments are required to find a reason for the different expression levels. Expression levels of the regulators FNR and the two component systems NarXL and NarQP as well as the nitrate/nitrite transporters would be interesting to analyse. In addition, different methods, such as ion-selective electrode (ISE) or mass spectrometry, are required to accurately determine the ammonium concentrations from chemostat cultures. An ammonia sensor, similar to the DO-probes that could be directly attached to the chemostat, would be ideal. Furthermore, it would be interesting to measure NO directly to see if they correlate with the N₂O levels. For instance, a chemoluminescence NO analyzer could be used (Molstad et al., 2007). In order to answer the question of whether high N₂O production is associated with pathogenicity, further organisms have to be tested. An interesting pathogen to study would be *Haemophilus influenzae*, a small non-motile Gram-negative bacterium of the *Pasteurellaceae* family. It is well characterised and its whole genome has been sequenced. However, the main reason is that it lacks the cytoplasmic Nar.

4 Investigating the existence of a nitrous oxide reductase in *Escherichia coli* and *Salmonella*

4.1 Introduction

Denitrification, the respiratory process that converts nitrate or nitrite to the gaseous forms NO, N₂O and N₂, is found among a broad range of microorganisms (Zumft, 1997). As discussed in the previous chapters, each reduction step is accomplished by different types of metalloenzyme. The last step of denitrification, the conversion of N₂O to N₂, is restricted to only some bacteria, archaea and fungi that possess the gene coding for a N₂O reductase (N₂OR). The denitrification pathway of enterobacteria like *E. coli* for instance is believed to terminate at N₂O and can therefore be called partial or truncated denitrification (Arkenberg et al., 2011). NosZ of *Paracoccus denitrificans* and other denitrifying bacteria, encoded by the highly conserved nosCRZDFYLX gene cluster, is the only bacterial enzyme that is known to possess N₂OR activity (Zumft and Kroneck, 2007). It is a copper-dependent periplasmic enzyme that contains two multi-copper centres; Cu_A and the catalytic Cu_Z (Pomowski et al., 2011). Some hypotheses have been made that other bacterial and archaeal organisms, including *E. coli*, *Yersinia kristensii*, *Buttiauxella agrestis* and *Pyrobaculum aerophilum* are able to reduce N₂O by using alternative enzymes to NosZ, but no recognizable homologs have been found yet (Fernandes et al., 2010, Zumft and Kroneck, 2007, Kaldorf et al., 1993). It almost seems preposterous that among the great diversity of organisms, which have very versatile metabolic systems and that possess multiple homologs of enzymes with the same function, only one N₂OR gene cluster exists. Kaldorf et al. (1993) first suggested N₂OR activity in *E. coli*, but were not able to identify the enzyme catalyzing the reduction of N₂O to N₂ and failed to convince the community of its existence. In their study, they argued that N₂OR activity has not been previously shown for Enterobacteriaceae because the reduction of N₂O to N₂ requires high amounts of N₂O, which could not be generated by NO₂⁻ reduction in previous experiments. Additionally, NO₂⁻ shows an inhibitory effect on this reaction (Kaldorf et al., 1993). Chemostat experiments from our investigations (Chapter 3, Figures 16-18B) support the hypothesis that *E. coli* might possess a yet undiscovered N₂OR. Under nitrate-limited/glycerol-sufficient growth conditions, it was notable that the decline in N₂O after its peak

was steeper at the beginning and flattens towards the end of the experiment. The same behaviour can be observed in *Salmonella* chemostat cultures that have been run under the same conditions (Rowley et al., 2012). The possibility that gas is leaking out of the system has also been considered but was judged as unlikely as the decline in N₂O was too irregular. However, as mentioned in section 3.5.3, if N₂OR activity does exist, its reduction potential would be relatively weak with a reduction rate of approximately 0.2 nM N₂O mg⁻¹ min⁻¹ (Chapter 3, Figure 18B). Combining the findings from this study (Chapter 3) with that of Kaldorf *et al.* (1993), there are sufficient signs for the possible existence of N₂OR activity in *E. coli* and this will be further investigated in this chapter.

Different methods exist to test for N₂OR activity and involve the measurement of N₂, N₂O or the N₂/N₂O product ratio of the two denitrification metabolites. However, it is very difficult to quantify N₂ directly due to its high ambient concentration in the atmosphere (Yu et al., 2010). Direct measurements of N₂ often require ¹⁵N-enrichment techniques, special and expensive equipment and intensive preparations, such as helium flushing the growth vessels to get rid of atmospheric N₂. An indirect approach is to quantify N₂O by using an N₂OR inhibitor. The principle behind this is that N₂O accumulates in large quantities, which can be easily determined by GC analysis. Assuming that *E. coli* possesses a weak N₂OR, the amount of N₂O produced during nitrate respiration should increase when applying this inhibition technique. The most common technique to inhibit N₂OR activity and to assay the denitrification potential of soil is addition of acetylene (C₂H₂) to the cultures (Balderston et al., 1976, Yoshinari and Knowles, 1976, Zumft, 1997). Although acetylene does not exclusively block the activity of N₂OR, but also NO₃⁻ and NO₂⁻ reduction, it is still the most selective inhibitor (Zumft and Kroneck, 2007). The inhibitory effect on the reduction of NO₃⁻ or NO₂⁻ was shown to be only minimal, if C₂H₂ affect these processes at all (Yoshinari and Knowles, 1976, Kaldorf et al., 1993). The inhibition by C₂H₂ is reversible but its mechanism of action remains to be identified (Zumft, 1997). Nitrous oxide reductase activity can also be inhibited by low pH, resulting in a higher N₂O/N₂ product ratio (Bergaust et al., 2010). In *Paracoccus*, it has been shown that N₂OR is more affected at a low pH than any of the other reductases involved in denitrification and that its activity is

practically zero at pH 6 (Simek et al., 2002, Thomsen et al., 1994, Liu et al., 2010, Bergaust et al., 2010). The reason for this has been shown to be at the post-translational level, as all reductases showed lower transcription levels at a lower pH and impairment of the assembly of N₂OR in the periplasm has been suggested (Bergaust et al., 2010). These observations have all been made in *P. denitrificans* and might not apply to other organisms. Nonetheless, the pH test was used as a method in this chapter to test for a putative N₂OR in *E. coli*. The validity of this method is arguable if used on its own but it can be used in combination with results from other methods. The third method used in this study, to test for a N₂OR in *E. coli*, was to determine a N₂O reduction potential with the help of a modified Clark electrode. Since the electrode responds to O₂ as well as N₂O, an oxygen scrubbing system (glucose, glucose oxidase, catalase) has been used to maintain anaerobiosis throughout the experiment (Field et al., 2008, Englander et al., 1987).

The attempt to identify the enzyme that possesses N₂OR activity, assuming it exists, has also been covered in this chapter. CueO (also known as Yack or CuiD), a multicopper oxidase (MCO) located in the periplasm, was used as a starting point in this study. Another MCO, McoP, from the hyperthermophilic archaeon *Pyrobaculum aerophilum* has been proposed to possess N₂O reductase activity (Fernandes et al., 2010). Sequence alignments of McoP with CueO from *E. coli* and CotA from *Bacillus subtilis* indicated similarities and therefore support the hypothesis that CueO could possess a N₂OR activity under anaerobic conditions. Kaldorf *et al.* (1993) suggested that the catalysing enzyme is most likely to be a Cu-type enzyme due to its inhibition by acetylene. They further suggested that cytochromes most likely do not participate in the generation of reductants for N₂-formation in *E. coli*, as in contrast to denitrifiers azide did not block N₂O reduction. This description fits astonishingly well to the periplasmic CueO, which possesses four copper centres; one type 1 Cu, one type 2 Cu and two type 3 Cu that have been structurally characterised (Sakurai and Kataoka, 2007, Singh et al., 2011). CueO belongs to the CueR regulon which also comprises the membrane bound copper efflux pump CopA (Achard et al., 2010). This system is known as the copper efflux (cue) system. In contrast to *Salmonella*, *E. coli* possesses an additional system, the copper-

sensing (cus) system, which contributes to resistance at high (mM) copper concentrations. CueO is mainly involved in copper homeostasis and protection against metal ion-triggered oxidative stress (Fenton reaction; Fe^{2+} or $\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+}$ or $\text{Cu}^{2+} + \text{OH}^- + \text{OH}\cdot$), where toxic Cu(I) is oxidized to the less toxic Cu(II) in an O_2 dependent reaction (Partridge et al., 2007). It also shows cuprous oxidase ($\text{Cu}^+ \rightarrow \text{Cu}^{2+}$), ferroxidase ($\text{Fe}^{2+} \rightarrow \text{Fe}^{3+}$) and polyphenol oxidase activities. Although CueO is not involved in copper protection under anaerobic conditions (Outten et al., 2001), it is still possible that it has a yet unknown N_2OR activity during anaerobiosis. Furthermore, CueO is known to be required for systemic virulence of *Salmonella* in mice (Achard et al., 2010).

4.2 Aim

The aim of this study was to test the hypothesis that *E. coli* and *Salmonella* possess a nitrous oxide reductase and to identify the putative enzyme.

4.3 Experimental design

4.3.1 Anaerobic Hungate batch supplemented with acetylene

S. Typhimurium SL1344 and *E. coli* MG1655 were cultured anaerobically in Hungate tubes for 24 hours as described in section 2.7. The MGN media was supplemented with 22 mM nitrate and 5 mM glycerol to achieve nitrate-sufficient/glycerol-limited growth conditions. Hungate tubes, containing the minimal media, were sparged with acetylene for 3 min and left to equilibrate over night before they were inoculated with the respective cultures. *P. denitrificans* 1222 was used as control organism and was cultured in a similar way. However, *Paracoccus* minimal medium (Appendix A) was used instead of MGN and 5% (v/v) overnight culture (section 2.2.2) instead of the usual 2% was used as inoculum. The $\text{OD}_{590\text{nm}}$ has been determined spectrophotometrically as described in section 2.9. A separate reference was used for OD measurements

of *Paracoccus* cultures to account for differences between the two minimal media. N₂O was determined by GC analysis as described in section 2.11. Pure acetylene has also been measured with the GC to ensure that it did not influence the N₂O measurements of the culture samples, which contain N₂O in the form of a N₂O/acetylene mixture. The data result from three technical replicates.

4.3.2 Anaerobic Hungate batch at different pH levels

S. Typhimurium SL1344 and *E. coli* MG1655 were cultured anaerobically in Hungate tubes for 24 hours as described in section 2.7. The MGN media was supplemented with 22 mM nitrate and 5 mM glycerol to achieve nitrate-sufficient/glycerol-limited growth conditions. The organisms were cultured in different MGN media ranging from pH 5-9, but all overnight cultures were grown in MGN media with a pH of 6.7 (not adjusted). Alkaline media (pH 8 and 9) was prepared by the addition of the necessary amount of NaOH, while acidic media (pH 5 and 6) was adjusted to the desired pH by the addition of HCl. The OD_{590nm} has been determined spectrophotometrically as described in section 2.9. N₂O was determined by GC analysis as described in section 2.11. The experiment was performed in duplicate.

4.3.3 Anaerobic Hungate batch of Δ cueO cultures

The cultures were grown as described in section 4.3.1 but in the absence of acetylene. Data are mean \pm standard error (n=3).

4.3.4 N₂O electrode

The experiment was performed as described in section 2.12, using a modified Clark electrode. N₂O reduction rates were determined as described in Appendix C and are given in (Table 12). The saturated N₂O solution (19.33 \pm 2.78 mM) was prepared as described in section 2.12.4. The determined value agrees with the value found in the literature (25mM at 25°C and 1atm) (Kristjansson and Hollocher, 1980). The tested strains were *P. denitrificans* 1222, *Salmonella*

SL1344 WT, SL1344 $\Delta cueO$ and SL1344 $\Delta NsrR$. *Paracoccus* was used as positive control while dead cells, sterile media were and pH 6 media were used as negative control.

4.3.5 Mutant constructs

Salmonella SL1344 and *E. coli* MG1655 knock-out mutants, lacking the *cueO* gene, have been created by the λ -red method (Datsenko and Wanner, 2000) as described in section 2.15 and Table 4. The Plasmid pKD 4 (1700 bp) was used to create a Kanamycin resistant *cueO* mutant in *E. coli*, whereas pKD 3 (1300 bp) was used to create a Chloramphenicol resistant *Salmonella cueO* mutant. However, the phage transduction step (section 2.15.4) was not performed for *E. coli* as no phage was available. Colony PCR (section 2.3.1) was used to confirm correct mutations, using external and internal verification primers as described in section 2.15.3 and Table 5. The respective gel electrophoresis images are shown in Figure 22. The $\Delta nsrR$ mutant used in this study was kindly provided by Dr Anke Arkenberg (Rowley Lab, strain collection).

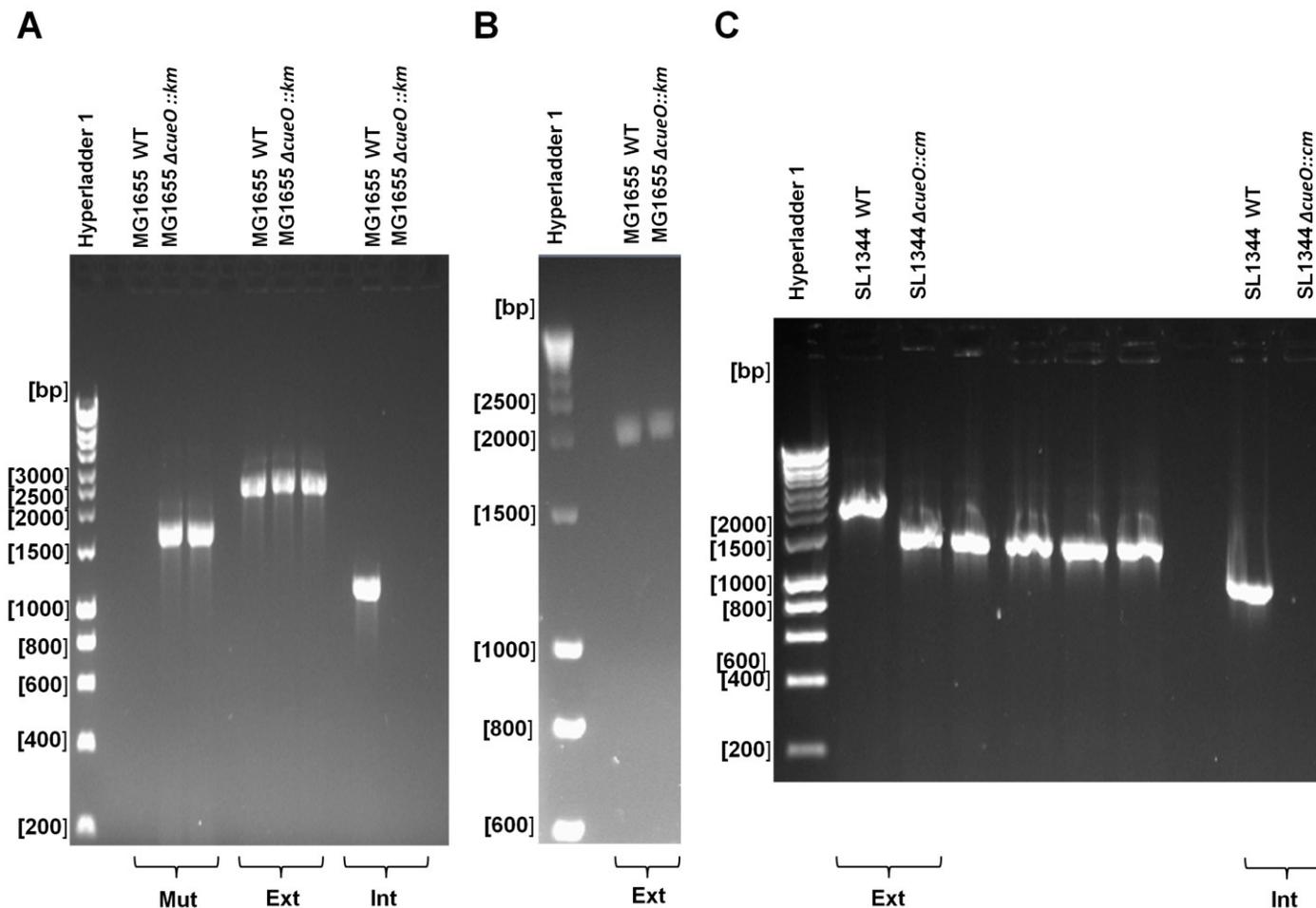


Figure 22 Agarose gel electrophoresis images of the PCR verification of *cueO* deletion mutants.

Hyperladder 1 (Bioline) was used as marker. Mut: mutation primer; Ext: external primer (\pm 50-400 bp up- and downstream of the gene of interest); Int: internal primer. Panel A (1% gel) and B (1.5% gel) show correct mutation of the *cueO* gene in *E. coli* MG1655, while Panel C (1% gel) confirms that the *Salmonella* SL1344 $\Delta cueO$ mutant is correct. Expected band sizes: MG1655 WT: Mut (no band), Ext (2150 bp), Int (1050 bp); MG1655 $\Delta cueO :: km$: Mut (1591 bp), Ext (2379 bp), Int (no band); SL1344 WT: Ext (1978 bp), Int (1007); SL1344 $\Delta cueO :: cm$: Ext (1667 bp), Int (no band).

4.4 Results

4.4.1 The effect of acetylene on N₂O production of nitrate-sufficient batch cultures

The first experiment to test the hypothesis that *E. coli* and *Salmonella* possess a yet unknown N₂O reductase was the addition of acetylene to the cultures. *P. denitrificans* Pd1222, *S. Typhimurium* SL1344 and *E. coli* MG1655 were cultured anaerobically in Hungate tubes for 24 hours. It is well known that *Paracoccus* uses the N₂OR NosZ to reduce N₂O to N₂ during denitrification. Thus, it was used as a positive control. With an OD_{max} of 0.6, Pd1222 WT achieved the highest growth values of all strains. Its OD_{max} was approximately twice of that of the MG1655 WT strain and approximately three times higher than that of the SL1344 WT strain (Figure 23A and Figure 24A). The addition of acetylene resulted in growth impairment of all tested strains, however, the growth of *Paracoccus* was least affected. After 24 hours post inoculation, the OD of the unmodified WT cultures (minus C₂H₂) Pd1222, MG1655 and SL1344 was approximately 20%, 60% and 80% higher compared to the respective cultures that had acetylene added to them. *Salmonella* struggled to survive acetylene addition and reached an OD_{max} of approximately 0.05. N₂O production at 24 hours post inoculation is expressed as absolute N₂O levels (Figure 23B and Figure 24B) and as N₂O per OD unit (Figure 23C and Figure 24C). *Paracoccus*, with a concentration of $3.2 \pm 0.67 \mu\text{M}/\text{OD units}$, was the lowest N₂O producing strain. However, it reached the highest N₂O levels when acetylene was added to the cultures. Under this condition, its N₂O levels increased by approximately three orders of magnitude, showing that acetylene successfully blocked the N₂OR NosZ. In *Salmonella*, the amount of N₂O produced per OD unit was approximately twice of that of the culture that was grown in the presence of acetylene (Figure 24C). Although, other than in Pd1222, the presence of acetylene resulted in lower N₂O levels per OD unit in the SL1344 strain, it is still a vast amount considering the fact that the culture had a severe growth defect.

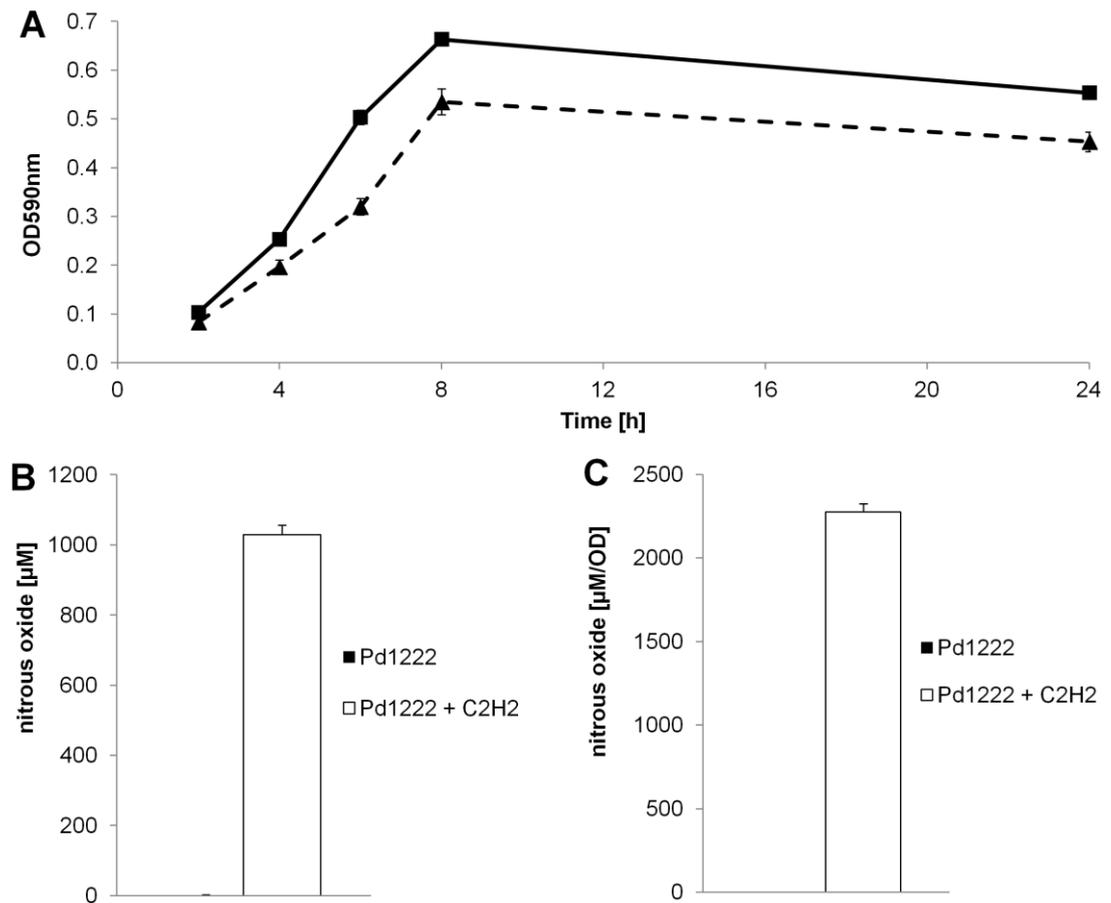


Figure 23 The effect of acetylene on growth and N₂O production of anaerobic batch cultures of *Paracoccus* during nitrate respiration.

Panel A: Anaerobic growth of *P. denitrificans* Pd1222 in Hungate tubes minus (square) and plus (triangle, dotted) the addition of acetylene (C₂H₂). Acetylene was added by sparging the Hungate tube for 3 min. OD_{590nm} was taken at 2, 4, 6, 8 and 24 hours post inoculation. The cultures were grown in 10 mL *Paracoccus* minimal medium (PMM), inoculated with 5% of a PMM overnight culture. Panel B and C: The nitrous oxide production (absolute levels: B [µM]; relative levels: C [µM/OD unit]) of *P. denitrificans* Pd1222 minus (black) and plus (white) C₂H₂ was measured after 24 hours post inoculation. Data are mean ± standard deviation (technical triplicate).

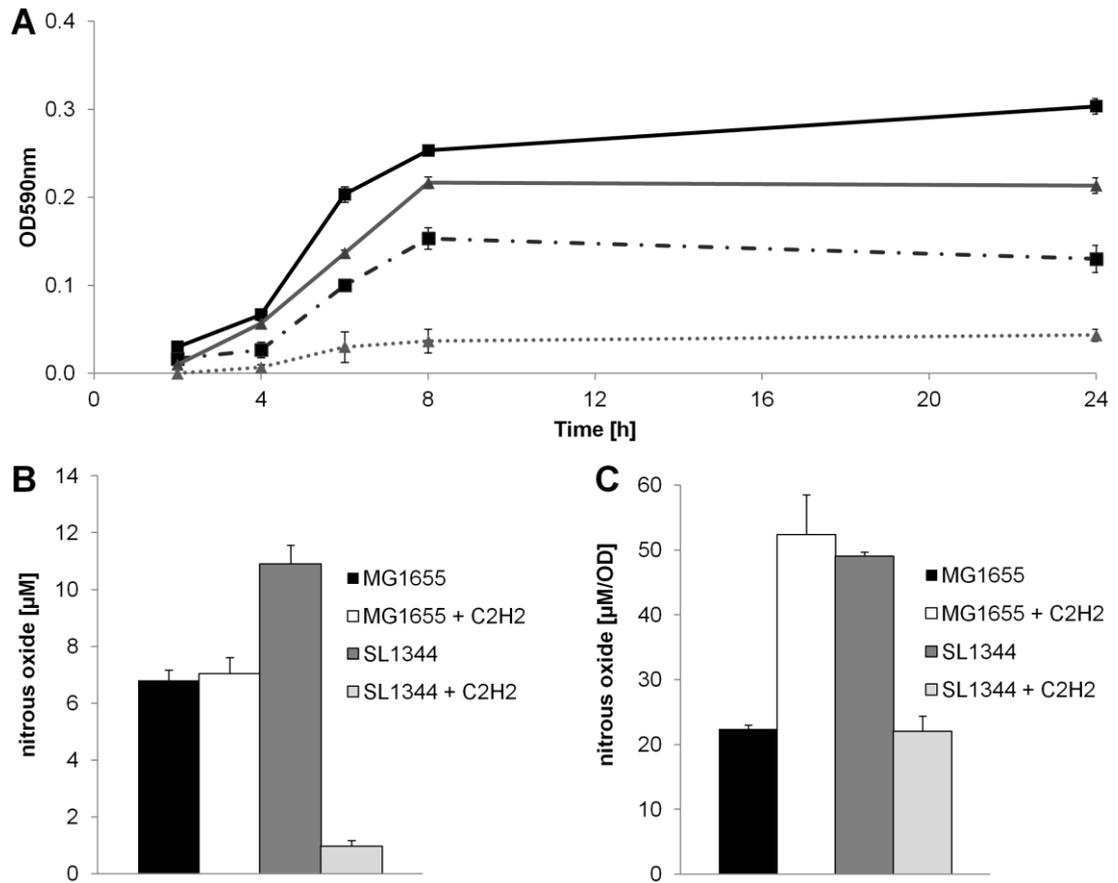


Figure 24 The effect of acetylene on growth and N₂O production of anaerobic batch cultures of *Salmonella* and *E. coli* during nitrate respiration.

Panel A: *Salmonella* SL1344 minus (square, black solid line) and plus (square, black dash dotted line) acetylene (C₂H₂); *E. coli* MG1655 minus (triangle, grey solid line) and plus (triangle, grey dotted line) C₂H₂. Acetylene was added by sparging the Hungate tubes for 3 min. OD_{590nm} was taken at 2, 4, 6, 8 and 24 hours post inoculation. The cultures were grown in 10 mL MGN (N⁺/G⁻) medium, inoculated with 2% of a MGN overnight. Panel B and C: The nitrous oxide production (absolute levels: B [µM]; relative levels: C [µM/OD unit]) of *Salmonella* SL1344 minus (black) and plus (white) C₂H₂ and *E. coli* MG1655 minus (dark grey) and plus (light grey) C₂H₂ was measured after 24 hours post inoculation. Data are mean ± standard deviation (technical triplicate).

Furthermore, the *Salmonella* SL1344 culture that had acetylene added to it produced the same amount of N₂O per OD unit compared to the unmodified *E. coli* MG1655 culture (Figure 24C), which had a six times higher OD at 24 hours post inoculation (Figure 24A). There is no detectable difference in the absolute N₂O levels of *E. coli* MG1655 cultures plus and minus acetylene (Figure 24B). However, if the growth difference is taken into account, the amount of N₂O per OD unit of MG1655 (-C₂H₂) is approximately 60% lower than that of MG1655 (+C₂H₂) (Figure 24C). The same test was performed with the *E. coli* W3110 strain and showed that even the absolute N₂O levels were approximately 13% higher in the culture that had acetylene added to it. Although the effect of acetylene on the N₂O production of *E. coli* and *Salmonella* is weaker compared to that in *Paracoccus*, it should be considered that we are looking for a N₂OR with a relatively weak activity (Chapter 3, Figure 18B). These results indicate the existence of a N₂OR in *Salmonella* and *E. coli* but further tests are necessary to confirm this, as acetylene does not exclusively block the activity of N₂OR but can also affect other enzymes involved in anaerobic nitrate respiration (Zumft and Kroneck, 2007).

4.4.2 The effect of pH on N₂O production of nitrate-sufficient batch cultures

In *Paracoccus* the N₂O reductase *NosZ* is dysfunctional at pH 6 and below, resulting in an accumulation of N₂O (Simek et al., 2002, Thomsen et al., 1994, Liu et al., 2010, Bergaust et al., 2010). To see if pH has the same effect on the N₂O levels in *E. coli*, *E. coli* MG1655 was cultured anaerobically in Hungate tubes for 24 hours at a pH range of 5-9. All overnight cultures were grown at pH 6.7. Acidic pH (pH 5 and 6) resulted in a slower growth rate and a lower OD_{max}, while alkaline pH (pH 8 and 9) resulted in a higher OD_{max} compared to almost neutral pH (pH 6.7) conditions (Figure 25A). Furthermore, it is noticeable that MG1655 needs longer to adapt to an alkaline pH shift (pH 6.7 of overnight culture to pH 8 or 9) compared to an acidic pH shift (pH 6.7 of overnight culture to pH 6 or 5) (Figure 25A 2-4h time span). The N₂O levels differed greatly at the different pH levels.

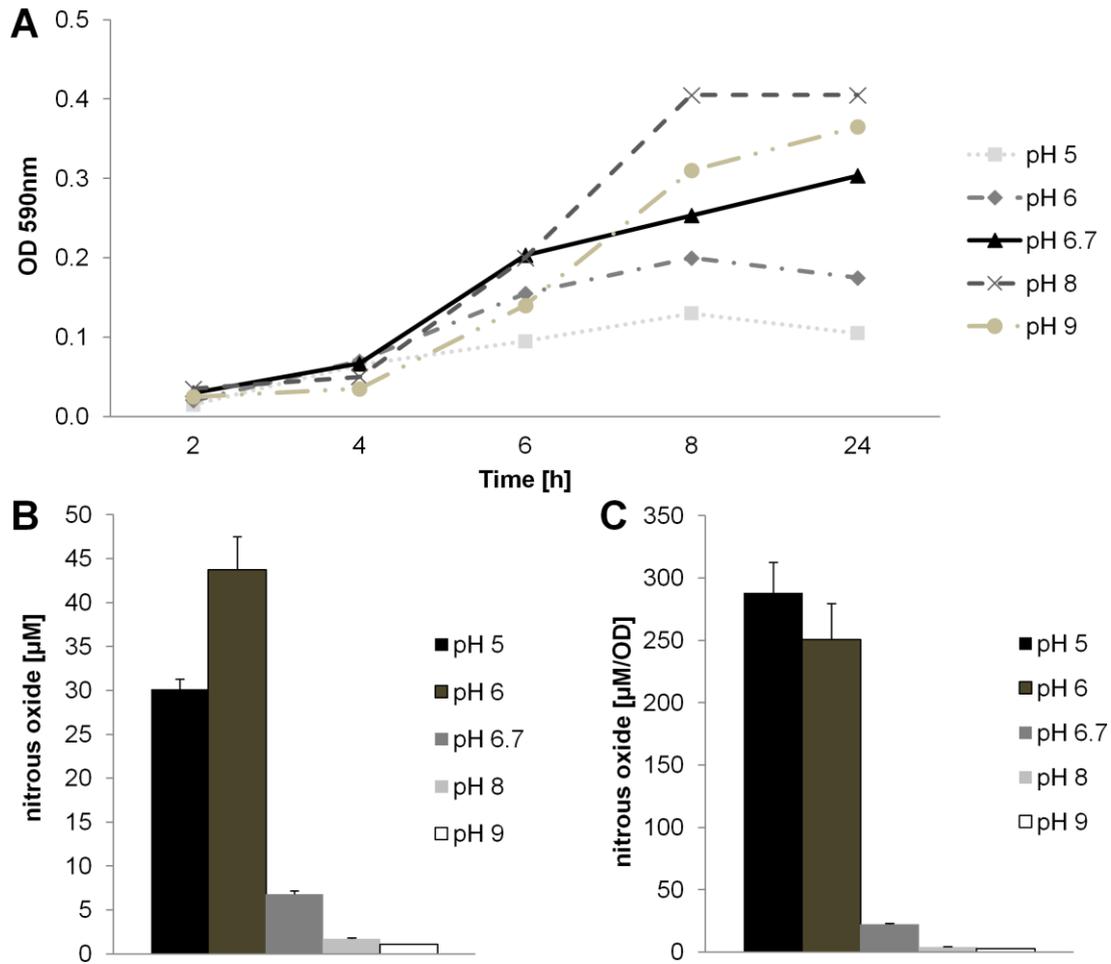


Figure 25 The effect of pH on growth and N₂O production of anaerobic batch cultures during nitrate respiration.

Panel A: Anaerobic growth of *E. coli* MG1655 in Hungate tubes at different pH levels; pH 5 (square, light grey, dotted), pH 6 (diamond, medium dark grey, dash dotted), pH 6.7 (triangle, black), pH 8 (cross, dark grey dashed) and pH 9 (circle, ochre, long dash dot dot). The cultures were grown in 10 mL MGN (N⁺/G⁻) medium, inoculated with 2% of a MGN overnight. All overnight were grown in pH 6.7 MGN (N⁺/G⁻) medium. OD_{590nm} was taken at 0, 2, 4, 6, 8 and 24 hours post inoculation. Panel B and C: The nitrous oxide production (absolute levels: B [µM]; relative levels: C [µM/OD unit]) of *E. coli* MG1655 at pH 5 (black), pH 6 (brown), pH 6.7 (dark grey), pH 8 (light grey) and pH 9 (white) was measured after 24 hours post inoculation. Data are mean ± standard error (n=2).

The N₂O production at 24 hours post inoculation is expressed as absolute N₂O levels (Figure 25B) and as N₂O per OD unit (Figure 25C). N₂O production per OD unit was highest at acidic pH and lowest at an alkaline pH (Figure 25C). For instance, MG1655 cultures grown at an acidic pH produced approximately 12-15 times more N₂O per OD unit compared to pH 6.7 cultures and approximately 60-100 times more than cultures grown at an alkaline pH. A similar trend is seen when looking at the absolute N₂O levels (Figure 25B). Different pH had the same effect on *Salmonella* cultures, with the exception that *Salmonella* needed longer to adapt to pH 9. Similar to the acetylene test, these results could indicate the existence of a N₂OR in *Salmonella* and *E. coli* but further tests are necessary to confirm this, as it was shown that the maximum initial NO production rate of *E. coli* was measured at pH 5.5-6 (Ji and Hollocher, 1988).

4.4.3 N₂O production of nitrate-sufficient batch cultures of *Salmonella* and *E. coli* Δ *cueO* mutants

The results above further support the notion that *E. coli* and *Salmonella* possess weak N₂OR. A few indications in the literature led to the assumption that the multicopper oxidase CueO might possess a yet unknown N₂OR activity under anaerobic conditions (see introduction). Therefore, an *E. coli* MG1655 Δ *cueO* mutant was constructed and cultured anaerobically in Hungate tubes for 24 hours. Surprisingly the Δ *cueO* mutant had a severe growth defect, reaching an OD_{max} of only 0.1 compared to the OD_{max} of 0.3 of the WT culture. It was noticeable that the mutant culture formed cell aggregations rather than growing homogeneously in suspension (Appendix D). Furthermore, after 24 hours many cells of the Δ *cueO* mutant culture had precipitated from the medium. A possible explanation is given by Tree *et al.* (2007), who showed that a mutation of *cueO* in *E. coli* MG1655 leads to A) a decrease in expression of genes associated with motility and to B) an increased expression of genes associated with autoaggregation (Tree *et al.*, 2007). However, in the aerobic overnight culture there was no detectable difference between the WT (OD value: 0.439) and the mutant strain (OD value: 0.430) and both Hungate batch cultures had the same amount of bacteria (cfu) when plated out. Next, the N₂O levels of the Δ *cueO* mutant were compared to that of the WT culture. Assuming that CueO has

N₂OR activity, the mutant strain should have higher N₂O levels. The N₂O production at 24 hours post inoculation is expressed as absolute N₂O levels (Figure 26B) and as N₂O per OD unit (Figure 26C). The absolute N₂O levels were approximately 35% higher in the $\Delta cueO$ mutant compared to the WT, whereas the amount of N₂O per OD unit was even approximately 70% higher. However, as many of the $\Delta cueO$ mutant cells have precipitated after 24 hours but were shown to be alive, the Hungate tubes were inverted for a new OD measurement after the gas sample has been taken. The OD of the inverted tubes was the same for both strains. Thus, to get a more precise comparison of the amount of N₂O per OD unit between the WT and the mutant strain, the values of the inverted tubes were used for calculation. Consequently, the difference of the amount of N₂O per OD between both strains is 35%, exactly the same as the absolute N₂O levels.

Although, the higher N₂O level of the $\Delta cueO$ mutant would suggest that CueO has a weak N₂O reductase activity under this condition, this result has to be looked at critically due to the mutant's growth defect. Therefore, a *Salmonella* SL1344 $\Delta cueO$ mutant was constructed and tested under the same conditions. In contrast to *E. coli*, the mutation of *cueO* did not result in a growth defect in *Salmonella* (Figure 27A). The *Salmonella cueO* mutant had the same N₂O levels as the WT (Figure 27B and C), indicating that the difference seen in *E. coli* is most likely due to its growth defect rather than N₂OR activity. In order to exclude a copper requirement for correct functioning in CueO, as it is found in the NosZ of *Paracoccus*, the experiment was repeated with the addition of 1 μ M CuSO₄. However, the addition of copper resulted in a slight growth delay and had no effect on N₂O production of the $\Delta cueO$ mutants.

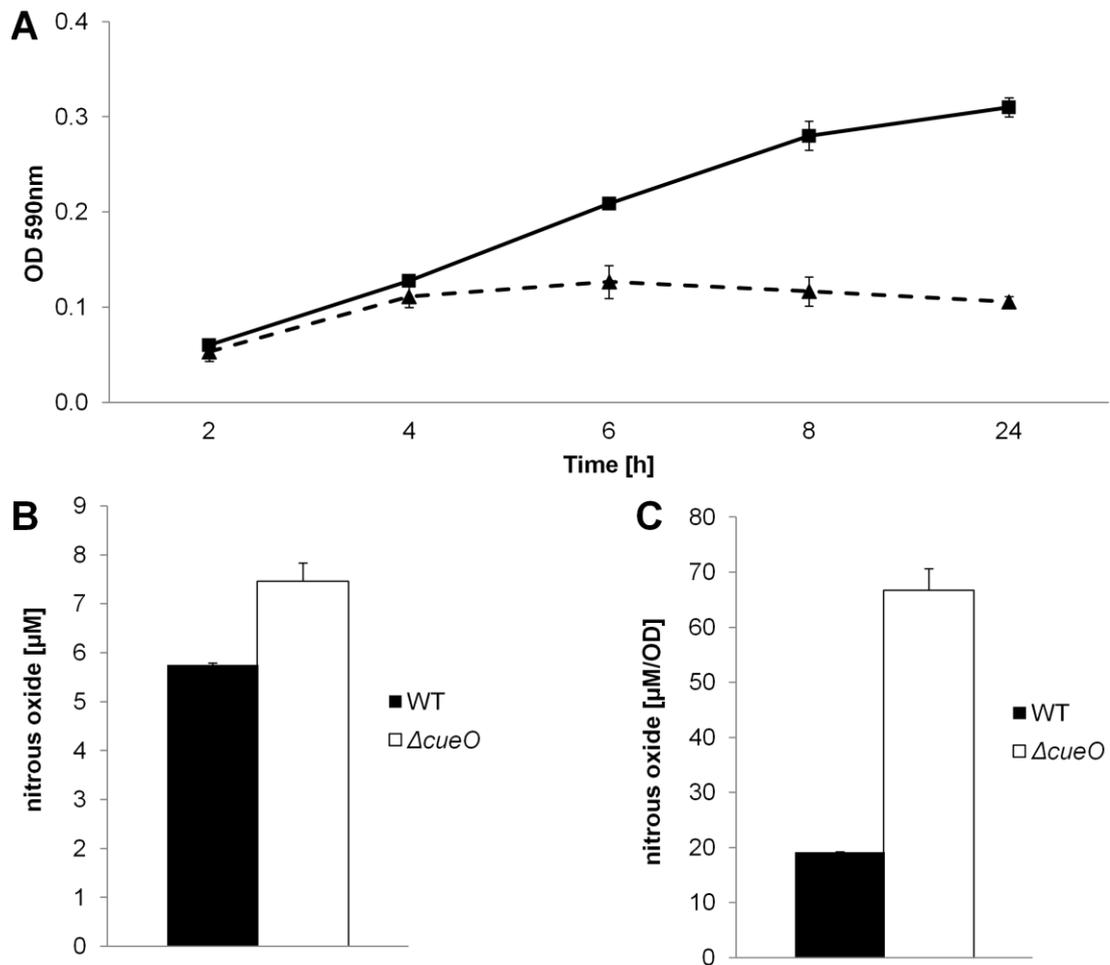


Figure 26 Growth and N₂O production of anaerobic batch cultures of *E. coli* WT vs. $\Delta cueO$ during nitrate respiration.

Panel A: Anaerobic growth of *E. coli* MG1655 WT (square) and *E. coli* MG1655 $\Delta cueO$ (triangle, dashed) in Hungate tubes. OD_{590nm} was taken at 2, 4, 6, 8 and 24 hours post inoculation. The cultures were grown in 10 mL MGN (N⁺/G⁻) medium, inoculated with 2% of a MGN overnight. Data are mean ± standard deviation (n=3). Panel B and C: The nitrous oxide production (absolute levels: B [μM]; relative levels: C [μM/OD unit]) of *E. coli* WT (black) and *E. coli* $\Delta cueO$ (white) was measured after 24 hours post inoculation. Data are mean ± standard error (n=3).

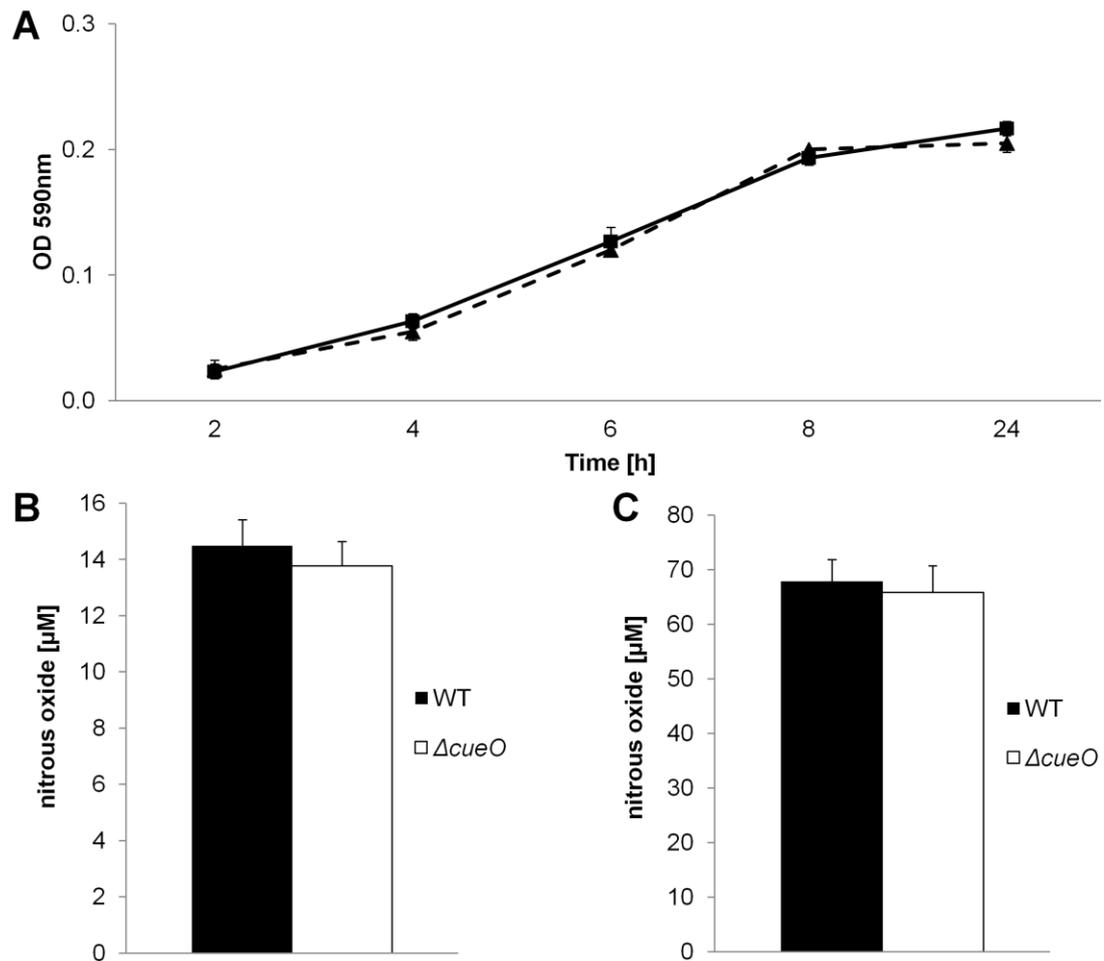


Figure 27 Growth and N₂O production of anaerobic batch cultures of *S. Typhimurium* WT vs. $\Delta cueO$ during nitrate respiration.

Panel A: Anaerobic growth of *S. Typhimurium* SL1344 WT (square) and *S. Typhimurium* SL1344 $\Delta cueO$ (triangle, dashed) in Hungate tubes. OD_{590nm} was taken at 2, 4, 6, 8 and 24 hours post inoculation. The cultures were grown in 10 mL MGN (N⁺/G⁻) medium, inoculated with 2% of a MGN overnight. Data are mean \pm standard deviation (n=3). Panel B and C: The nitrous oxide production (absolute levels: B [μM]; relative levels: C [$\mu\text{M}/\text{OD unit}$]) of *S. Typhimurium* WT (black) and *S. Typhimurium* $\Delta cueO$ (white) was measured after 24 hours post inoculation. Data are mean \pm standard error (n=3).

4.4.4 N₂O consumption in *Salmonella*

The last method employed to test for the existence of a N₂OR was the N₂O electrode (modified Clark electrode) assay. The experiment was carried out in 2 mM nitrate-free MGN media. *Salmonella* was used as the model organism due to the growth defect of the *E. coli cueO* mutant. Since the electrode responded to O₂ in addition to N₂O, an O₂ scrubbing system was applied to maintain anaerobiosis. Once anaerobic, 2 x 300 µL of saturated N₂O solution was added to the electrode chamber, which resulted in an increase in signal (Figure 28). Since the output signal of the Clark electrode is given in oxygen units, it has to be altered so that the apparent amount of O₂ can be converted to the amount of N₂O in solution (see Appendix C). The reaction was initiated by the addition of 200 µL of concentrated cells, leading to a sudden drop in signal (Figure 28). In order to calculate the N₂O reduction rate of the injected cells, the background rate (before injection of cells) had to be subtracted from the measured rate (after injection of cells). Figure 28 is a representative illustration of the N₂O reduction potential of *Salmonella* SL1344 WT. The results (± standard error) of all tested strains and experimental conditions are given in (Table 12). An example of the original electrode output graph for each strain and experimental condition, as well as an example calculation for the N₂O reduction rates (qcN₂O in Table 12) is given in Appendix C. *Paracoccus* Pd1222 was used as a positive control as it possesses the N₂OR NosZ, while autoclaved cells and media were used as negative controls. Initial tests with Pd1222 and SL1344 WT were performed in nitrate-sufficient media at 20°C to see if the experiment was working. Pd1222 had the highest N₂O reduction rate, which was approximately 50 times higher than that of SL1344 (Table 12). As N₂O reduction was also detectable in *Salmonella*, suggesting the presence of a N₂OR, further tests were performed. The experimental conditions were changed to nitrate-limitation and 37°C to have A) an optimal growth temperature for *Salmonella* and B) comparable N₂O reduction rates to the one calculated from nitrate-limited chemostat experiments (Chapter 3, Figure 18B).

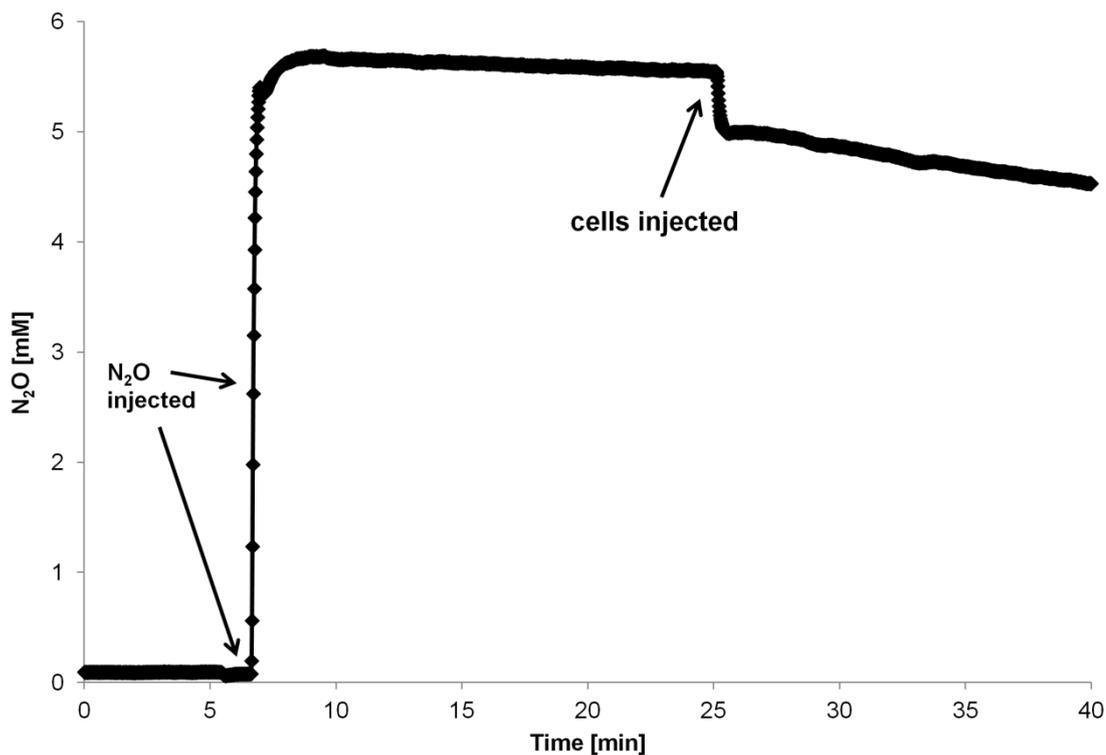


Figure 28 N₂O reduction in *Salmonella* SL1344.

A modified Clark electrode (Oxytherm) was used to determine the N₂O reduction rate of *Salmonella* SL1344. The assay was carried out in 2 mM nitrate-free MGN media, supplemented with 20 mM glycerol, in a stirred reaction chamber at 37°C. An enzymatic O₂ scrubbing system, consisting of glucose (16 mM), glucose oxidase (4 units/mL) and catalase (20 units/mL) final concentration, was used to make the reaction chamber anaerobic. A saturated N₂O solution was added to the system. The reaction was initiated by adding 33.89 mg of cells. The background rate (before injection of cell) was subtracted from the measured rate (after injection of cells) to calculate the N₂O reduction rate of SL1344. The result (\pm standard error) are given in Table 12.

The N₂O reduction rate of SL1344 WT at 37°C was approximately seven times higher than that of SL1344 WT performed at 20°C. This result, together with the fact that N₂O was not reduced by any of the negative controls and neither by SL1344 cultures grown at pH6, strongly supports the hypothesis that *Salmonella* does possess weak N₂OR activity. The reduction potential of the SL1344 Δ *cueO* mutant was also tested. Since it matched the rate (within errors) of the WT strain, the result confirmed that CueO is most likely not a N₂OR. We then tested a Δ *nsrR* mutant, as NsrR is known to regulate many of the enzymes involved in anaerobic nitrate respiration. Surprisingly, besides the relatively big error bars, a lower N₂O reduction rate could be detected in the Δ *nsrR* mutant. This suggests that the N₂O reductase of *Salmonella* might be NsrR regulated. This hypothesis is tested in the next chapters.

Table 12 N₂O reduction rates (qcN₂O) using a modified Clark electrode.

Sample	Strain/ Condition	Glycerol NO ₃ ⁻ [mM]		Temp. [°C]	qcN ₂ O [μmol * mg ⁻¹ * min ⁻¹]
<i>P. denitrificans</i>	Pd1222*	5	22	20	3.20 ¹
<i>S. Typhimurium</i>	SL1344	5	22	20	0.07 ¹
<i>S. Typhimurium</i>	SL1344	20	5	37	0.51 ± 0.04
<i>S. Typhimurium</i>	SL1344 Δ <i>NsrR</i>	20	5	37	0.34 ± 0.11
<i>S. Typhimurium</i>	SL1344 Δ <i>cueO</i>	20	5	37	0.45 ± 0.02 ²
<i>S. Typhimurium</i>	SL1344 @ pH6	20	5	37	0.02 ± 0.01
Autoclaved cells	SL1344 *	20	5	37	0.00 ± 0.00
Media	n/a*	20	5	37	0.00 ± 0.00
NO₃⁻ free Media	n/a*	20	0	37	0.00 ± 0.00

*: control sample (Pd1222 = +ve; autoclaved cells and media = -ve); ¹: experiment performed once (n=1); ²: experiment performed in duplicate (n=2). All other data are mean ± standard error (n=3).

4.5 Discussion

The aim of this chapter was to test the hypothesis that *Salmonella* and *E. coli* possess a nitrous oxide reductase and to identify the putative enzyme. The experimental strategy consisted of a combination of different N₂OR inhibition assays, gene knock-out and nitrous oxide reduction potential experiments.

4.5.1 N₂OR inhibition experiments

Two approaches were used to test for the presence of a N₂OR in *Salmonella* and *E. coli*; acetylene inhibition and inhibition by low pH (\leq pH 6). Both conditions have been shown to block the N₂OR activity of *Paracoccus*' NosZ (Balderston et al., 1976, Yoshinari and Knowles, 1976, Zumft, 1997, Simek et al., 2002, Thomsen et al., 1994, Liu et al., 2010, Bergaust et al., 2010). In agreement with previous studies, the results of the acetylene experiment showed that the NosZ activity of *Paracoccus* was inhibited in the presence of acetylene. This was reflected in the N₂O levels that increased by approximately three orders of magnitude in the presence of acetylene compared to a very low accumulation of N₂O during its absence (Figure 23C). The presence of acetylene did not only result in higher N₂O levels but also in growth impairment (Figure 23A). The growth impairment was also detected in *E. coli* and *Salmonella*, but the impact was much more severe (Figure 24A). This would suggest that other enzymes apart from the N₂OR are affected by acetylene. Although former studies have shown that the effect of acetylene on the activity of enzymes other than the N₂OR is only minimal (Yoshinari and Knowles, 1976, Kaldorf et al., 1993, Zumft and Kroneck, 2007), our results do not disagree with them as the concentration of acetylene added to the system was very different. While the acetylene concentration used in most other studies was commonly between 5% and 10%, the concentration used in this study was presumably much higher, as the culture media was flushed with pure acetylene for three minutes. The growth impairment effect of acetylene has also been described in other organisms before. For instance, 10% of acetylene resulted in a slight growth impairment of the sulphate-respiring bacterium *Desulfovibrio gigas*,

while 5% did not (Payne and Grant, 1982). Acetylene that has been added to the head space of soil samples at a partial pressure of 10 Pa, impaired the growth of ammonia oxidizing archaea by inhibiting the *amo* gene, essential for nitrification (Offre et al., 2009). This shows that although acetylene is the most selective inhibitor for NosZ, it does affect other enzymes, especially if present in very high concentrations and the effect might vary extremely between different organisms. Further studies are necessary to elucidate the mechanism of growth inhibition.

The presence of acetylene resulted in an increase of the N₂O levels per OD unit in *E. coli* MG1655 by approximately 60% (Figure 24C) and it increased the absolute N₂O levels of *E. coli* W3110 by approximately 13%. Even *Salmonella*, where growth has been inhibited almost completely by the presence of acetylene, produced a relatively high amount of N₂O, considering its poor growth conditions (Figure 24C). Thus, these results indicate the existence of a relatively weak N₂OR in *Salmonella* and *E. coli*, compared to *Paracoccus*' NosZ. Nonetheless, these results need to be interpreted carefully due to the growth impairment and further tests are required for validation.

The second approach to test for the existence of a N₂OR in *Salmonella* and *E. coli* was the effect of low pH. The results showed that the accumulation of N₂O decreased with an increasing pH (Figure 25C). This agrees with studies in *Paracoccus*, which showed that higher N₂O levels at increased pH are a result of altered N₂OR activity (Simek et al., 2002, Thomsen et al., 1994, Liu et al., 2010, Bergaust et al., 2010). Furthermore, these studies revealed that acidic pH has the biggest effect on N₂OR activity compared to other reductases involved in denitrification and that its activity was close to zero at pH 6. An explanation for this was given by Bergaust *et al.* (2010), who demonstrated that the pH affects the N₂OR post-translationally and that it most likely interferes with the assembly/folding of the enzyme due to its periplasmic location. This theory is consistent with studies made in *Paracoccus* and *E. coli*, which showed that the periplasm is likely more affected by external pH than the cytoplasm, since both organisms were able to maintain a cytoplasmic pH near 7 irrespective of the external pH (Booth, 1985, Wilks and Slonczewski, 2007). Assuming these

results are correct, the nitrate reductase NarG of *E. coli* and *Salmonella* is likely to be unaffected by external/periplasmic pH, since its active site is located in the cytoplasm. The same should be true for the cytoplasmic HmpA and NorVW (Figure 3). Therefore, N₂O is being produced but does not get reduced, resulting in higher N₂O levels. Nonetheless, a former *E. coli* study showed that initial rates of NO production in *E. coli* were maximal at a pH of 5.5-6 (Ji and Hollocher, 1988). Therefore, the higher N₂O levels produced from *E. coli* cultures that were grown at an acidic pH could be due to either side of the pathway; higher NO production or inhibition of N₂O reduction (Figure 25C). That being said, the results from the pH test must be interpreted with care and should only be considered in combination with other tests, because pH shifts of this degree affect the whole organism and not only the enzymes involved in anaerobic nitrate respiration. This was also reflected in the growth patterns, as growth increased with increasing pH (Figure 25A). The reason for this need to be elucidated, but *E. coli* appears to do a trade-off between high biomass versus N₂O production. Nonetheless, the combined results of the acetylene and the pH test indicate the existence of a weak N₂O reductase in *E. coli* and *Salmonella*.

4.5.2 Search for the N₂O reductase enzyme

Two approaches were used to A) confirm the findings from the N₂OR inhibition experiments and to B) find the putative N₂OR enzyme. Since a few indications in the literature led to the assumption that the multicopper oxidase CueO might possess a yet unknown N₂OR activity under anaerobic conditions (see introduction), *cueO* knock-out mutants were constructed in *E. coli* and *Salmonella*. Although mutation of *cueO* resulted in higher N₂O levels in *E. coli* (Figure 26B and C), which means that it is possible that CueO is a N₂OR, this result must be interpreted with care as *E. coli* had a severe growth defect (Figure 26A). It could be argued that these results are valid as the cells were alive and were simply less mobile due to autoaggregation and a decrease expression of motility genes Tree *et al.* (2007), but the *Salmonella* results suggested a different interpretation (Figure 27). The *Salmonella* Δ *cueO* mutant did produce the same amount of N₂O compared to the WT strain and had the

same growth pattern. Therefore, CueO is most likely not a N₂OR. This was confirmed by the N₂O electrode experiments, as the SL1344 $\Delta cueO$ mutant had a similar nitrous oxide reduction potential compared to the WT (Table 12). Furthermore, the electrode data confirmed the hypothesis of the N₂OR inhibition experiments that *Salmonella* does possess a relatively weak N₂O reductase, compared to *Paracoccus*' NosZ. Thus, it is likely to be the same for *E. coli*.

Although the enzyme could not be identified, a decreased reduction rate in the $\Delta nsrR$ mutant suggests that the N₂OR of *Salmonella* might be regulated by NsrR. This hypothesis is plausible, considering the fact that NsrR is a global regulator (Table 9). Therefore, the contribution of the NsrR regulon to nitrous oxide emissions has been examined in the next two chapters.

4.6 Future work

Since the high amount of acetylene used in this study resulted in severe growth impairments in *E. coli* and *Salmonella*, it would be interesting to see if the presence of lower acetylene concentrations would still result in higher N₂O levels, without affecting the cultures' growth. Furthermore, a combined approach of transcriptional studies (qRT-PCR or microarray) and enzyme kinetics (Methyl viologen assay or protein film voltammetry) could provide clarification on whether the higher N₂O levels are a result of higher NO production or an impairment of N₂O reduction. The same techniques would be very useful for pH experiments. Since the *E. coli* MG1655 $\Delta cueO$ mutant had a severe growth defect, a new *cueO* mutant should be designed in the closely related *E. coli* strain W3110. Another interesting experiment would be to combine the acetylene experiment with the N₂O electrode assay. The rationale is that acetylene, added after cells have been injected, should decrease or zero the N₂O reduction rate. Furthermore, ¹⁵N enrichment experiments could be used to see if N₂ is produced by *Salmonella*, which would be expected as N₂O reduction has been detected. Although the results from this chapter suggest the presence of an N₂OR in *Salmonella* and *E. coli*, further tests are required to identify the enzyme.

**5 The NsrR regulon of *Salmonella* SL1344 Δ *nsrR*
during anaerobic nitrate respiration**

5.1 Introduction

Salmonella and *E. coli* possess many enzymes that provide protection against reactive nitrogen species, such as the cytotoxin nitric oxide, generated either as part of the innate immune response or as product of their own metabolism. As described in Chapter 1, NorR and NsrR are two regulators of *Salmonella* and *E. coli*, known to be able to sense NO directly. NorR senses NO via a mononuclear non-heme iron centre (D'Autréaux et al., 2005) and activates transcription of *norVW* upon NO exposure. In contrast, NsrR senses NO directly via a [2Fe-2S] cluster and is known to play a central role in nitrosative stress response as a global repressor (Rodionov et al., 2005, Bodenmiller and Spiro, 2006, Gilberthorpe et al., 2007, Tucker et al., 2008, Karlinsey et al., 2012, Filenko et al., 2007). The presence of NO leads to nitrosylation of the cluster and loss of DNA binding capacity and hence derepression of NsrR regulated genes (Bodenmiller and Spiro, 2006, Tucker et al., 2008). This was also shown in macrophage experiments, where *nsrR* transcription was reduced as soon as NO has been produced by the iNOS of the macrophage at eight hours post infection (Eriksson et al., 2000, Hammarlof et al., 2013, Gilberthorpe et al., 2007). Originally, NsrR was identified in the nitrifying bacterium *Nitrosomonas europaea* as a nitrite-sensing repressor (Beaumont et al., 2004) before it was shown to be the major NO-responsive regulator among a wide range of bacterial taxa, including *E. coli* (Bodenmiller and Spiro, 2006), *S. Typhimurium* (Gilberthorpe et al., 2007), *Bacillus subtilis* (Nakano et al., 2006), *Neisseria gonorrhoeae* and *Neisseria meningitidis* (Overton et al., 2006, Heurlier et al., 2008). By using computational modelling, Rodionov *et al.* (2005) predicted that *hcp*, *hcr*, *hmpA*, *ytfE*, *ygbA* and a *tehB* homolog are regulated by NsrR. These have been confirmed by transcriptional studies in *Salmonella* and *E. coli* and additional genes including *yeaR*, *yoaG* and the *Salmonella* specific *STM1808* have been identified (Bang et al., 2006, Filenko et al., 2007, Lin et al., 2007, Gilberthorpe et al., 2007, Bodenmiller and Spiro, 2006, Karlinsey et al., 2012). NsrR was found to be the principal regulator of *hmpA* transcription in *Salmonella* and HmpA was identified to be the most conserved member of the NsrR regulon (Rodionov et al., 2005, Bodenmiller and Spiro, 2006). The

periplasmic Nap and Nrf belong as well to the NsrR regulon, but not their cytoplasmic counterparts. In total the NsrR regulon comprises at least 60 genes (Tucker et al., 2010). In *E. coli*, at least 20 genes are repressed by NsrR, while a similar amount were found to be activated (Filenko et al., 2007). Although the NsrR regulon has been widely determined, the contribution of many members, including *hcp*, *hcr*, *ytfE*, *ygbA*, *yeaR*, *yoaG* and *STM1808*, to nitrosative stress resistance has been poorly characterised (Table 13). Hcp-Hcr have a predicted function in NO detoxification under both aerobic (Karlinsey et al., 2012) and anaerobic conditions (Cole, 2012). YtfE is known to be important for the iron-sulphur cluster repair of NO damaged proteins, but the exact repair mechanism still needs to be elucidated (Constantinidou et al., 2006, Overton et al., 2008, Justino et al., 2007). The other NO responsive operons are associated with tellurite resistance (*yeaR-yoaG*, *STM1808*), aerobic NO detoxification (*STM1808*) and unknown function (*ygbA*) (Table 13). Furthermore, most NsrR studies have been performed in *E. coli* rather than in *Salmonella* and were aimed to look at the response to exogenic NO sources. Others have been performed aerobically, which seems not ideal considering the fact that nitrate respiration and NO detoxification are predominantly anaerobic processes. Thus, this study was performed to determine the contribution of members of the NsrR regulon to N₂O production in *Salmonella* under both nitrate-sufficient and nitrate-limited conditions. In this chapter, a combined physiological (chemostat) and transcriptional approach (qRT-PCR and Microarray analysis) was used to further characterize the NsrR regulon. Based on this, some of the interesting and less characterised NsrR regulon members were then tested in Chapter 6 for their contribution to N₂O production using mutagenesis experiments.

5.2 Aim

The aim of this study was A) to investigate the contribution of NsrR to endogenous N₂O production in *Salmonella* and B) to further determine and analyze the NsrR regulon in order to identify the function of less characterised NsrR regulon members during anaerobic nitrate respiration.

Table 13 NsrR repressed genes with limited knowledge about their function in nitrosative stress defence.

Gene	Main Function	References
<i>STM1808</i>	Putative role in NO detoxification	(Karlinsky et al., 2012)
<i>ytfE</i>	Repair of [Fe-S] clusters	(Efromovich et al., 2008, Karlinsky et al., 2012, Justino et al., 2007, Overton et al., 2008, Pullan et al., 2007, Bodenmiller and Spiro, 2006)
<i>hmpA</i>	NO detoxification	(Poole et al., 1996, Filenko et al., 2007, Poole, 2005, Membrillo-Hernandez et al., 1998, Karlinsky et al., 2012, Pullan et al., 2007, Bang et al., 2006)
<i>ygbA</i>	Unknown, putative role in NO ₃ ⁻ respiration	(Karlinsky et al., 2012, Gilberthorpe et al., 2007, Rodionov et al., 2005, Bodenmiller and Spiro, 2006, Filenko et al., 2007)
<i>yeaR-yoaG</i>	Putative role in tellurite resistance and nitrosative stress defence	(Lin et al., 2007, Filenko et al., 2007, Karlinsky et al., 2012, Squire et al., 2009, Vine and Cole, 2011)
<i>tehB*</i>	Putative role in NO detoxification ; Tellurite resistance	(Karlinsky et al., 2012, Bodenmiller and Spiro, 2006, Gilberthorpe et al., 2007, Rodionov et al., 2005, Partridge et al., 2009, Justino et al., 2005b, Turner et al., 1997, Whitby et al., 2010)
<i>hcp</i>	NH ₂ OH/NO responsive, controversial	(Filenko et al., 2005, Chismon et al., 2010, Tucker et al., 2010, Karlinsky et al., 2012, Constantinidou et al., 2006, Wolfe et al., 2002, Filenko et al., 2007)
<i>hcr</i>	NH ₂ OH/NO responsive, controversial	(Filenko et al., 2005, Chismon et al., 2010, Tucker et al., 2010, Karlinsky et al., 2012, Constantinidou et al., 2006, Wolfe et al., 2002, Filenko et al., 2007)

* NsrR binding site has been identified but regulation has not been confirmed, yet.

5.3 Experimental design

5.3.1 Continuous chemostat cultures

S. Typhimurium SL1344 wild-type (WT) and SL1344 $\Delta nsrR$ were cultured in continuous chemostats as described in section 2.8. The SL1344 $\Delta nsrR$ strain was kindly provided by Dr Anke Arkenberg (lab culture collection). N_2O was determined by GC analysis as described in section 2.11. The MGN media was either supplemented with 22 mM nitrate and 5 mM glycerol to achieve nitrate-sufficient/glycerol-limited growth conditions or with 20 mM glycerol and 5 mM nitrate to achieve nitrate-limited/glycerol-sufficient growth. RNA extraction and qRT-PCR were performed as described in section 2.16.

5.3.2 Microarray

Microarray analysis of glycerol-sufficient/nitrate-limited continuous chemostat cultures of *S. Typhimurium* SL1344 WT and SL1344 $\Delta nsrR$ were performed as described in section 2.17. RNA was extracted as described in section 2.16. Total RNA (10 μ g) was reverse transcribed to cDNA using Affinity Script (StrataGene) and labelled by using random primers to incorporate the Cy5-dCTP (Amersham) fluorescent dye as described in section 2.17.1. The labelled cDNA was mixed (1/5) with 2 μ g of Cy3-dCTP labelled (Gibco Biorprime DNA labelling system) chromosomal DNA and hybridized onto an 8 x array Agilent slide as described in section 2.17.2-3. The microarray slides were scanned by a Genepix 4000A scanner and the scan was filtered and quantified with the Genepix Pro 7.0 software as described in section 2.17.4. The data were then normalised using the Batch Anti Banana Algorithm in R (BABAR) (Alston et al., 2010) before they were analyzed with the Gene Spring 7.3 (Agilent) software. A minimum cut-off threshold of 2-fold change was set for statistical significance. The experiment was performed in duplicate.

5.4 Results

5.4.1 Nitrate-sufficient chemostat cultures of SL1344 WT vs. $\Delta nsrR$

Salmonella SL1344 WT and $\Delta nsrR$ were cultured in continuous chemostats in order to compare their nitrate respiration products; nitrate, nitrite and N₂O. Their growth patterns (Figure 29A and Figure 30A) were similar to that seen in *E. coli* (Chapter 3, Figures 13-15A). Once the air supply was cut off, 22 hours post inoculation, a decline in biomass was detectable before a steady-state was reached. It seems that the steady-state was reached as early as 50 hours post inoculation, which would be much earlier than in *E. coli*. Furthermore, the OD_{max} of *Salmonella* is lower compared to that of the *E. coli* chemostat cultures. This was also reflected in the Hungate batch culture experiments (Figure 11) and is supported by our previous *Salmonella* study (Rowley et al., 2012). The SL1344 *nsrR* mutant did grow slightly worse in comparison to the *Salmonella* WT culture; reflected by a lower OD_{max} and a lower steady-state OD (Figure 29A and Figure 30A). The glycerol-sufficient chemostat experiments also supported this finding, as the WT strain reached a 2-fold higher OD_{max} during aerobic batch mode (Figure 32A and Figure 33A). A possible explanation for these data is given by Gilberthorpe *et al.* (2007). HmpA is able to cause toxic effects to the cells by the production of superoxide anion from O₂ (Membrillo-Hernandez et al., 1996, Wu et al., 2004). Therefore, elevated levels of HmpA in $\Delta nsrR$ during aerobic conditions (Figure 34) might result in oxidative stress and could in turn lead to a lower biomass.

Similar to *E. coli* (Chapter 3, Figures 13-15B), *Salmonella* started to respire nitrate once the culture was switched to continuous anaerobic mode at 22 hours post inoculation and nitrite accumulated almost stoichiometrically during the transition phase (22-48h) (Figure 29B). Afterwards, the nitrite became converted to N₂O, indicated by a decline in the nitrite concentration and the simultaneous increase in N₂O levels. In comparison with the *E. coli* chemostat cultures, *Salmonella* produced more N₂O. For instance, the rate of N₂O production was twice (qpN₂O: 0.68) that observed with MC1000, the highest

N₂O producing *E. coli* strain (qpN₂O: 0.38). This agrees with our previous study (Rowley et al., 2012) and was also reflected in the transcription levels (Figure 31), with all genes important for high N₂O production being up-regulated. The SL1344 *nsrR* mutant behaves very similarly to the WT strain but reaches even higher N₂O levels. The qp[N₂O] value for the $\Delta nsrR$ mutant (0.92) is approximately 25% higher compared to that of the WT, which is related to up-regulation of the NO detoxification systems, especially *hmpA* and *norV* (Figure 31). Elevated production of N₂O in $\Delta nsrR$ has also been detected in *Moraxella catarrhalis*, an aerobic human respiratory tract pathogen, and was shown to be a result of more rapid consumption of nitric oxide (Wang et al., 2008). The quicker consumption of NO was also demonstrated in aerobic *Salmonella* studies (Gilberthorpe et al., 2007, Karlinsey et al., 2012). However, this study demonstrates for the first time that a *Salmonella nsrR* mutant produces higher N₂O levels endogenously compared to the WT strain during anaerobic nitrate respiration. Since *napDA*, *nrfA* and *hmpA* are repressed by NsrR, they become derepressed in the *nsrR* mutant, resulting in higher expression levels compared to that of the WT (Figure 31). Surprisingly, the transcription of *narG*, *nirB* and *norV* was also increased in the *nsrR* mutant, although they do not belong to the NsrR regulon. Thus, it is possible that this is an indirect effect of the *nsrR* mutation. However, a counter-argument is that the expression levels of the *nsrR* mutant were also increased during the aerobic phase (Figure 31; W1, N1) and not only during anaerobiosis (Figure 31; W2, N2), which could indicate a direct effect. Since these data were obtained from a single chemostat experiment, replicate experiments are required for clarification. Nonetheless, transcriptional analysis of glycerol-sufficient chemostat cultures (Figure 34) suggested that the results are correct, as all tested genes apart from *norV* were up-regulated in the *nsrR* mutant.

SL1344

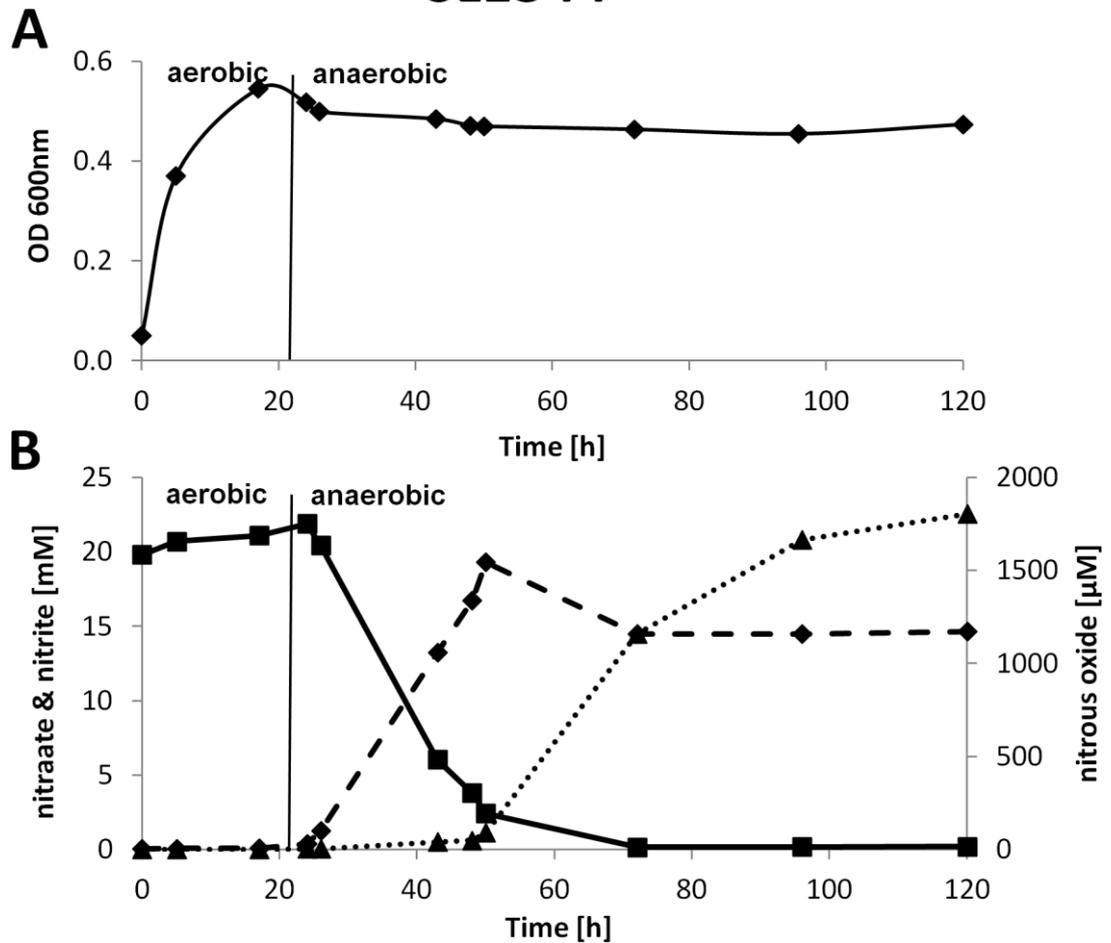


Figure 29 Nitrate consumption and nitrite and nitrous oxide production in a nitrate-sufficient/glycerol-limited continuous culture of *S. Typhimurium* SL1344.

The culture was run as described in Figures 13-15. The nitrate concentration in the feed reservoir was 22 mM and the glycerol concentration was 5 mM to simulate nitrate-sufficient growth. A: Biomass and B: nitrate (square symbols), nitrite (diamond symbols) and nitrous oxide (triangle symbols). The data are obtained from a single experiment.

SL1344 $\Delta nsrR$

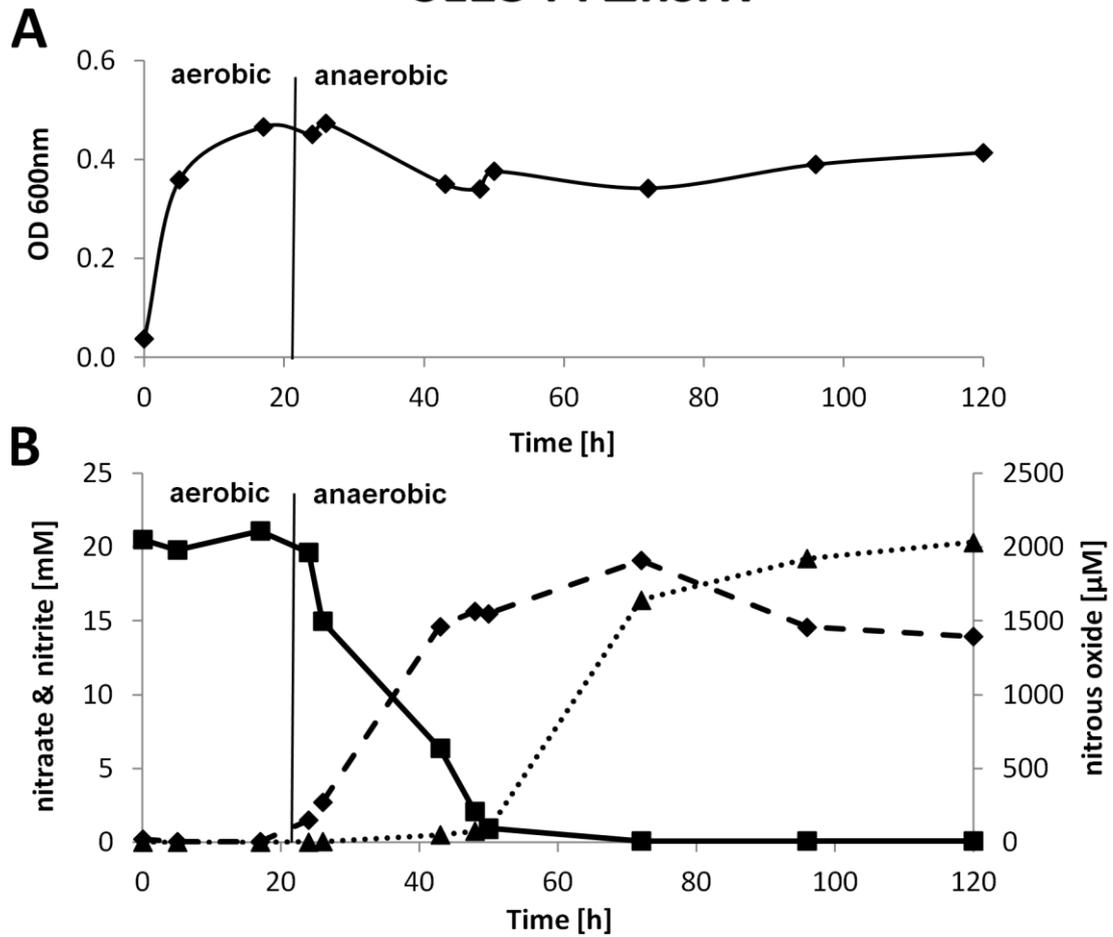


Figure 30 Nitrate consumption and nitrite and nitrous oxide production in a nitrate-sufficient/glycerol-limited continuous culture of *S. Typhimurium* SL1344 $\Delta NsrR$.

The culture was run as described in Figures 13-15. The nitrate concentration in the feed reservoir was 22 mM and the glycerol concentration was 5 mM to simulate nitrate-sufficient growth. A: Biomass and B: nitrate (square symbols), nitrite (diamond symbols) and nitrous oxide (triangle symbols). The data are obtained from a single experiment.

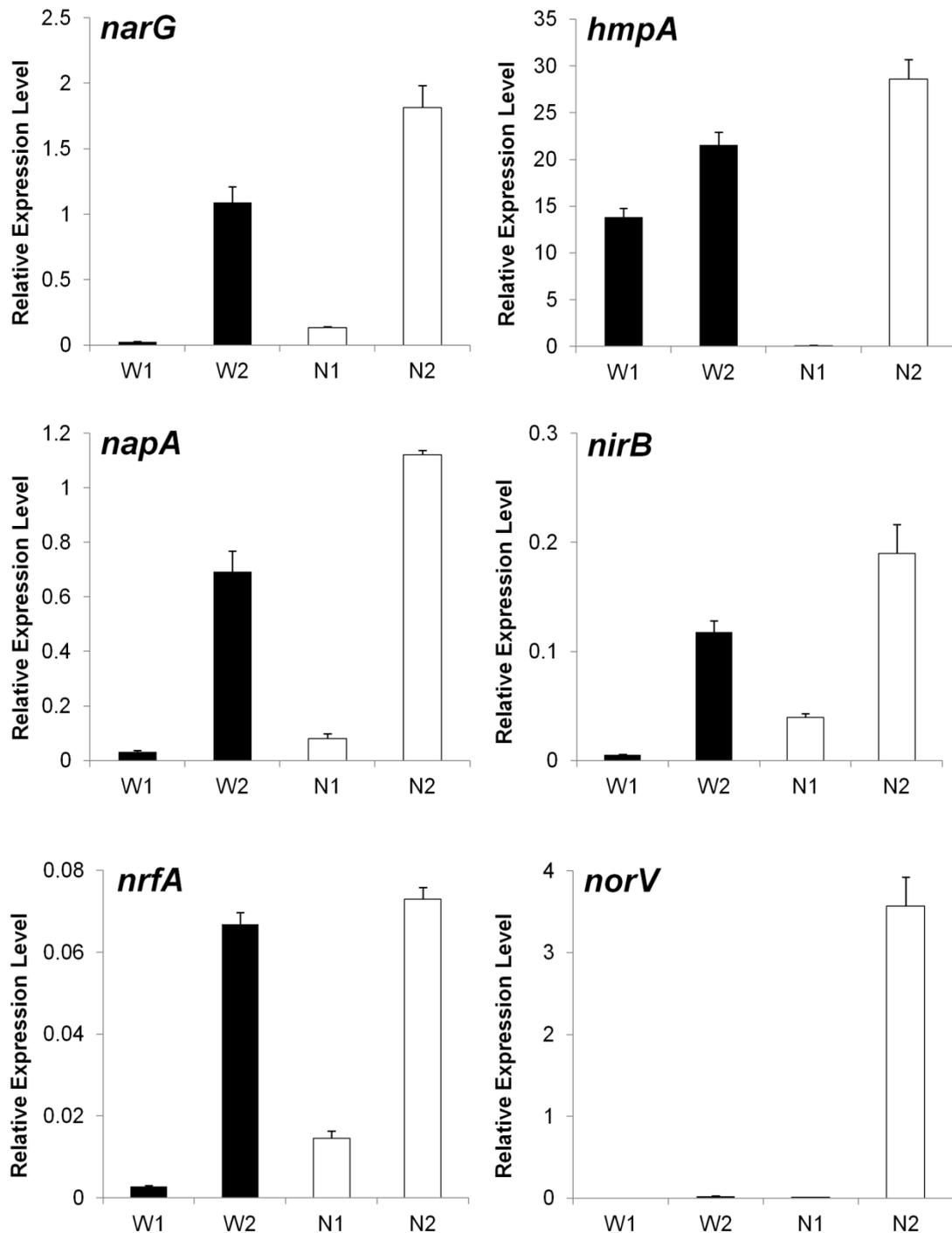


Figure 31 qRT-PCR of nitrate-sufficient/glycerol-limited continuous culture of *S. Typhimurium* SL1344 WT and $\Delta nsrR$.

The qRT-PCR experiment was performed in technical triplicates from one total RNA preparation. The calculated threshold cycle (Ct) for each gene was normalized to the Ct of the *ampD* control. The values report the relative expression levels of SL1344 WT (W) and $\Delta nsrR$ (N) at 5 h (1: aerobic) and 120 h (2: anaerobic) time-point. The data are obtained from a single chemostat experiment. The error bars result from qRT-PCR analysis performed as technical triplicates.

5.4.2 Nitrate-limited chemostat cultures of SL1344 WT vs. $\Delta nsrR$

The greater carbon availability during nitrate-limited/glycerol-sufficient growth (Figure 32A) resulted in a higher biomass during the aerobic growth phase in *Salmonella* SL1344 WT compared to nitrate-sufficient growth conditions (Figure 29A). However, in comparison with *E. coli* cultures (Figures 16-18A) the OD_{max} is approximately 2-fold lower, which agrees with the results of our previous *Salmonella* study (Rowley et al., 2012). Similar to *E. coli*, the steady-state biomass of N^-/G^+ and N^+/G^- *Salmonella* WT cultures was almost identical. As mentioned above, the *nsrR* mutant culture reached an OD_{max} that was only half of that of the WT strain; potentially as a result of the production of the highly reactive radical O_2^- by an over-expressed *hmpA* (McLean et al., 2010). The lower OD is not only seen during the aerobic growth phase but stretches across the entire experiment.

The nitrate respiration pattern of *Salmonella* WT resembled that of *E. coli*. Nitrate was consumed within a few hours after the switch to anaerobic continuous mode and nitrite, as well as nitrous oxide accumulated transiently during the transition phase. In the steady-state, nitrous oxide remained at approximately 5 μ M with a N_2O production rate (qp_{N_2O}) of 0.003 ± 0.001 . In contrast, nitrate respiration in the $\Delta nsrR$ strain resembled more that of a nitrate-sufficient chemostat culture. Once produced, the nitrite levels stayed relatively high throughout the experiment and decreased only very slowly; reaching a concentration of approximately 2.5 mM in the steady-state. Increased nitrite levels (not significantly) have also been detected in IFN- γ -stimulated macrophages that were infected with a *Salmonella nsrR* mutant (Gilberthorpe et al., 2007). In conjunction with high nitrite levels, N_2O accumulated to relatively high levels (150 μ M) during the transition phase and remained fairly level throughout the experiment. The qp_{N_2O} value of the SL1344 $\Delta nsrR$ (0.150 ± 0.015) was approximately 6-15 times higher than that of the low producing *E. coli* strains MG1655 and W3110, grown under N^+/G^- conditions, and only half of that of MC1000. In order to find an explanation for this, transcriptional analysis was performed.

SL1344

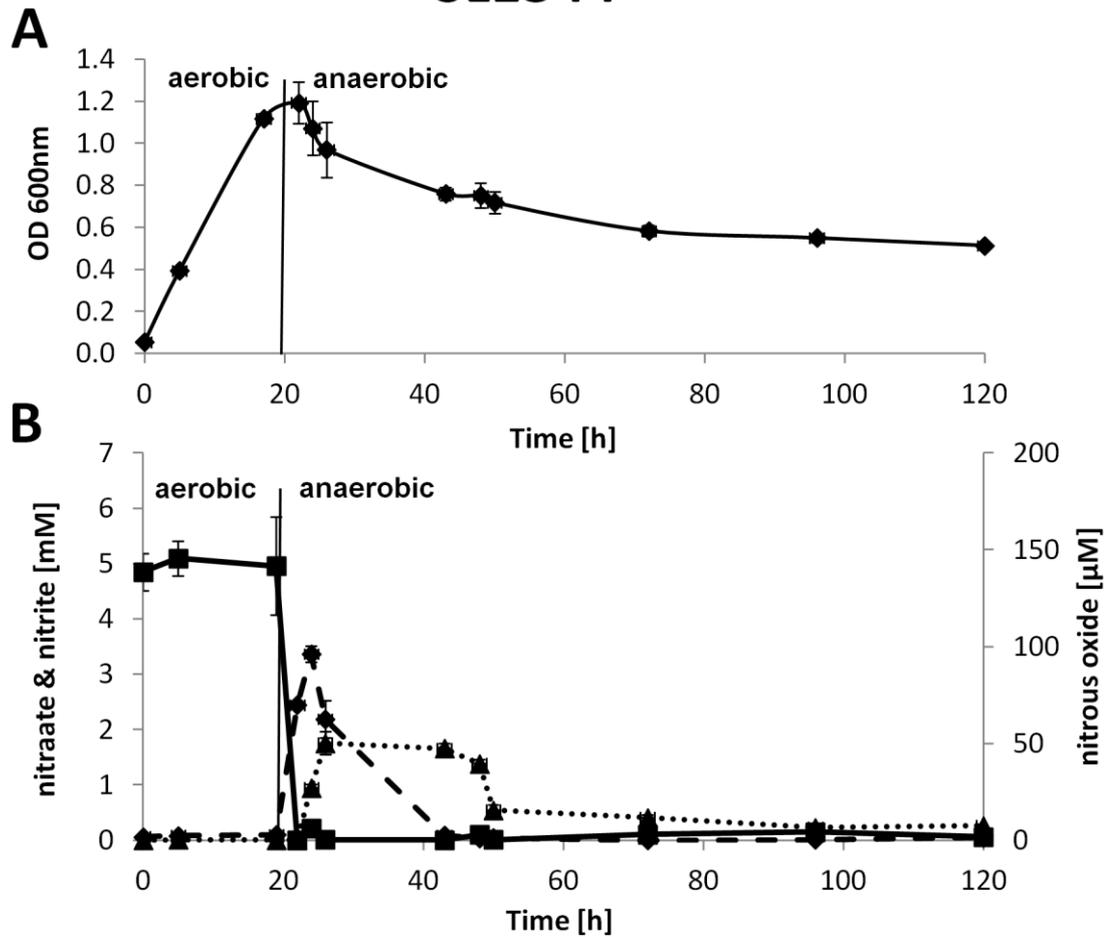


Figure 32 Nitrate consumption and nitrite and nitrous oxide production in a glycerol-sufficient/nitrate-limited continuous culture of *S. Typhimurium* SL1344.

The culture was run as described in Figures 13-15, but the air supply was cut off after 20 hours post inoculation. The glycerol concentration in the feed reservoir was 20 mM and the nitrate concentration was 5 mM to simulate glycerol-sufficient growth. A: Biomass and B: nitrate (square symbols), nitrite (diamond symbols) and nitrous oxide (triangle symbols). Data are mean \pm standard error (n=3).

SL1344 $\Delta nsrR$

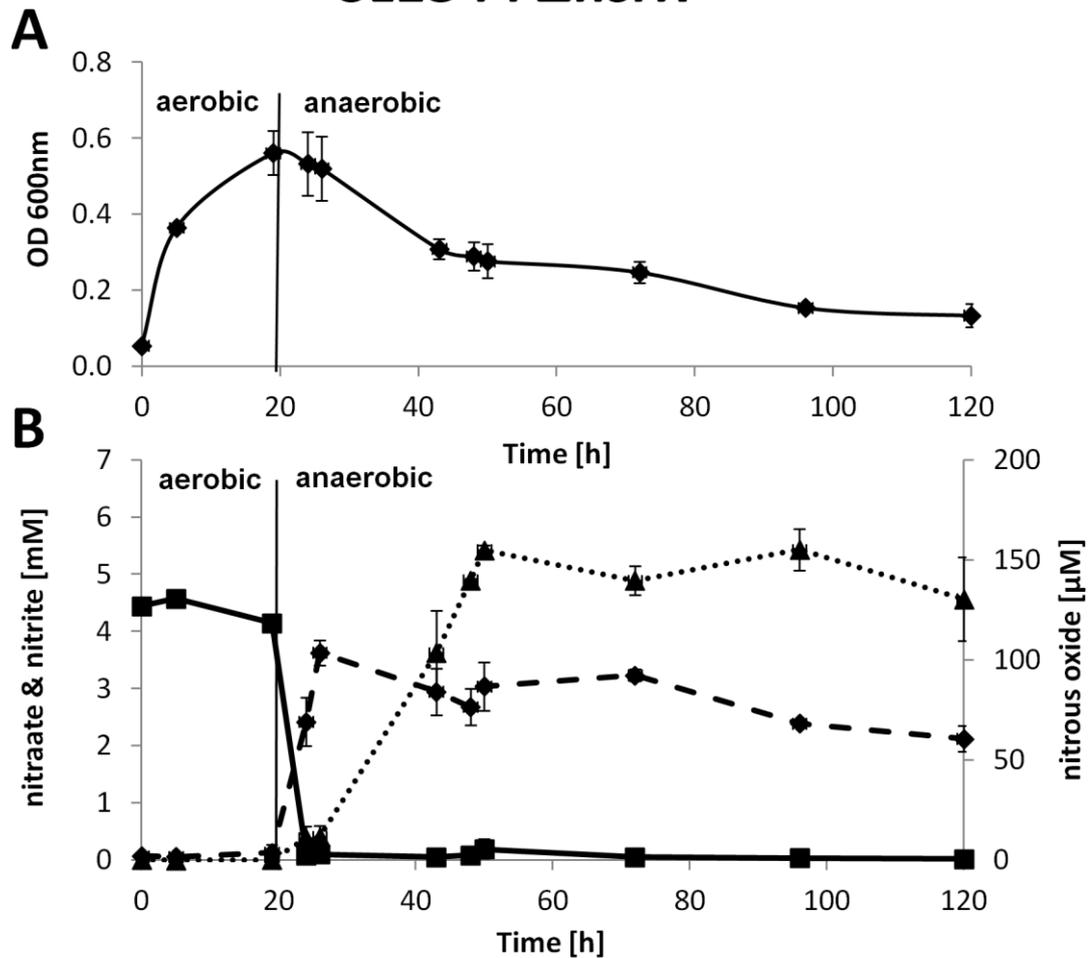


Figure 33 Nitrate consumption and nitrite and nitrous oxide production in a glycerol-sufficient/nitrate-limited continuous culture of *S. Typhimurium* SL1344 $\Delta nsrR$.

The culture was run as described in Figures 13-15, but the air supply was cut off after 20 hours post inoculation. The glycerol concentration in the feed reservoir was 20 mM and the nitrate concentration was 5 mM to simulate glycerol-sufficient growth. A: Biomass and B: nitrate (square symbols), nitrite (diamond symbols) and nitrous oxide (triangle symbols). Data are mean \pm standard error (n=3).

Similar to the transcription levels of the nitrate-sufficient chemostats, most of the nitrate respiration systems were highly expressed in the *nsrR* mutant, with *norV* being an exception. The genes that were most affected are *napA*, *hmpA* and *nirB*. Under anaerobic conditions, the relative expression level of *napA*, *nirB* and *hmpA* were approximately 6.5-, 20- and 150-fold higher in the $\Delta nsrR$ culture compared to the WT strain (Figure 34; W2, N2). While this was expected to be the case for *napA* and *hmpA*, as they are both repressed by NsrR, it was a surprise for *nirB*. As explained earlier, this might be due to an indirect effect. The same might be true for *narG*, which was activated during anaerobiosis in $\Delta nsrR$ but not in the WT strain. The higher expression levels of *nirB* and *narG* in the *nsrR* mutant were confirmed by microarray analysis (Appendix C and Table 16). Although, an up-regulation of *narG* and *hmpA* is atypical for nitrate-limited growth conditions, it could explain why the nitrate respiration pattern of the *nsrR* mutant resembles that of a nitrate-sufficient culture. Even though the general consensus is that HmpA has its main function during aerobic NO detoxification and plays only a minor role during anaerobiosis (Gardner and Gardner, 2002, Hutchings et al., 2002, Poock, 2002, Mills et al., 2008), there have been some reports that suggest its importance during anaerobic NO detoxification (Crawford and Goldberg, 1998, Gilberthorpe et al., 2007). The discrepancies were believed to be a result of different growth conditions. Since *norV* is only poorly expressed (Figure 34), it is likely that there is another enzyme that is responsible for the high N₂O levels seen in $\Delta nsrR$. Thus, it is plausible that HmpA plays a key role in NO detoxification and N₂O production during glycerol-sufficient growth conditions. This hypothesis is supported by Hungate batch culture experiments that showed that an *hmpA* mutant had significantly lower levels of N₂O compared to the WT under nitrate-limited conditions (Chapter 6; Figure 41). In addition, it is possible that NsrR is a positive regulator of a weak N₂OR that becomes inactivated in the absence of NsrR, resulting in an accumulation of N₂O (see Chapter 4). In order to further understand the $\Delta nsrR$ phenotype, a microarray has been performed under nitrate-limited/glycerol-sufficient conditions.

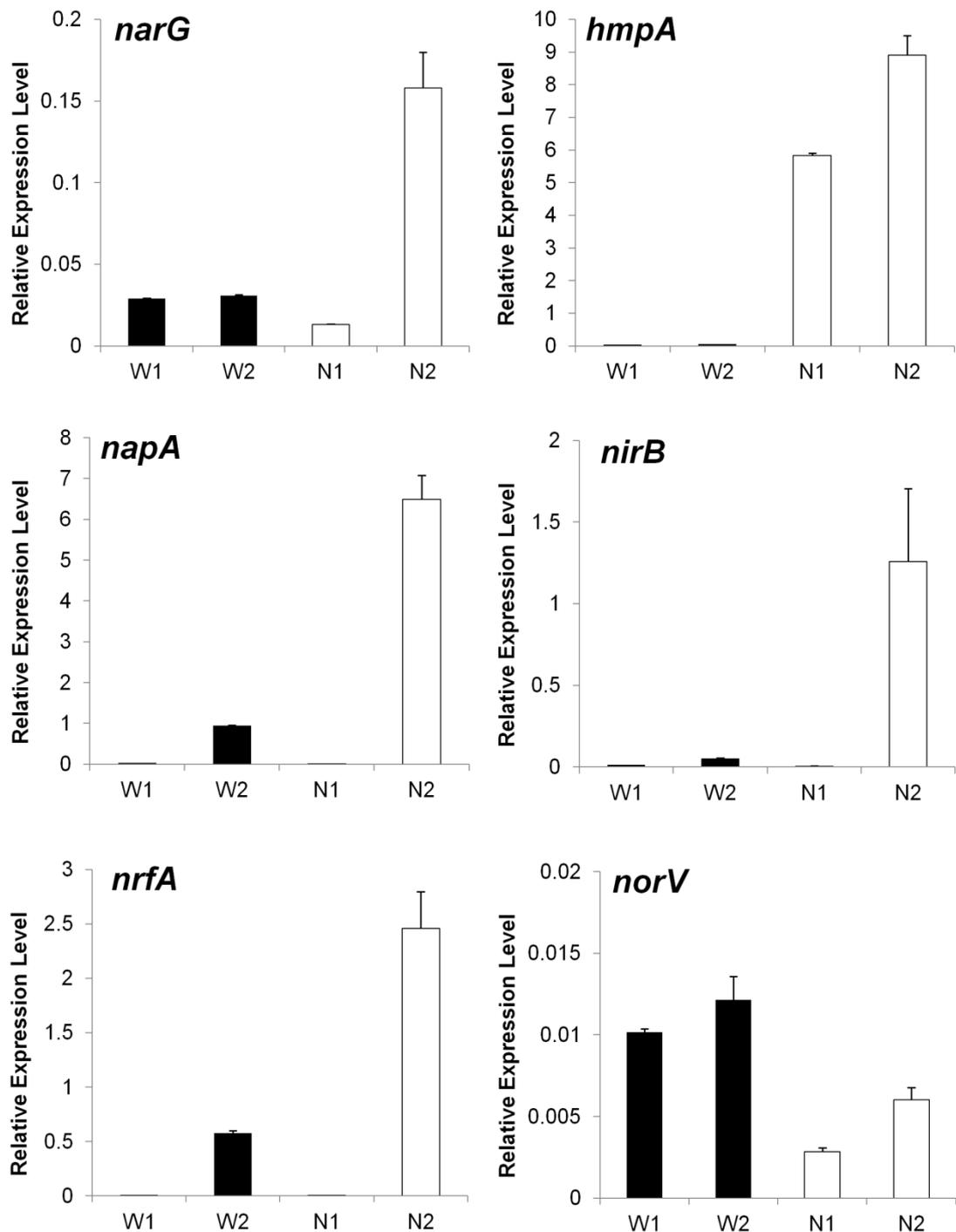


Figure 34 qRT-PCR of glycerol-sufficient/nitrate-limited continuous culture of *S. Typhimurium* SL1344 WT and $\Delta nsrR$.

The qRT-PCR experiment was performed in technical triplicates from one total RNA preparation. The calculated threshold cycle (Ct) for each gene was normalized to the Ct of the *ampD* control. The values report the relative expression levels of SL1344 WT (W) and $\Delta nsrR$ (N) at 5 h (1: aerobic) and 120 h (2: anaerobic) time-point. The data are obtained from a single chemostat experiment. The error bars result from qRT-PCR analysis performed as technical triplicates.

5.4.3 Microarray analysis of SL1344 WT vs. $\Delta nsrR$

DNA microarrays were used to measure the global transcriptional differences between glycerol-sufficient/nitrate-limited continuous chemostat cultures of *Salmonella* SL1344 WT and the *nsrR* mutant. A 2-fold cut-off threshold was applied to the data. Although RNA samples were taken from duplicate chemostat experiments, the aerobic data set of both strains (WT and the *nsrR* mutant) represent only a single chemostat run, due to hybridization problems. Nonetheless, the data were validated with a previous SL1344 WT microarray. Furthermore, genes involved in the anaerobic metabolism (Figure 35 and Figure 36) were up-regulated during anaerobiosis in both strains, *nsrR* expression was significantly decreased in the *nsrR* mutant strain compared to the WT strain and the transcription profile of all genes tested by qRT-PCR matches that of the microarray data. Thus, even though it would be necessary to repeat the microarray study for publication purposes, there is enough evidence that this data set is reliable.

When comparing the expression profile of the WT strain under aerobic vs. anaerobic conditions, 1391 genes (that match the 2-fold change cut-off threshold) were differently transcribed. 655 of these were up-regulated under anaerobic conditions and 726 of them were expressed at lower levels anaerobically. The amount of genes changing during the shift from aerobic to anaerobic growth in the *nsrR* mutant is very similar to that of the WT, with 1492 genes being differently transcribed. Of these, 725 genes were up-regulated under anaerobic conditions and 767 were down-regulated. Approximately 1/3 (291) of the genes that were up-regulated anaerobically in the WT strain were also up-regulated in the *nsrR* mutant. Slightly more genes were up-regulated anaerobically in the WT strain (374) that were not up-regulated in $\Delta nsrR$, while 434 genes were only up-regulated anaerobically in the *nsrR* mutant. Approximately half of the down-regulated genes (511) were lower expressed in both of the strains. The other half comprises 256 genes that were only down-regulated in the *nsrR* mutant and 215 genes that were exclusively down-regulated in the WT strain.

Of greatest interest to this study is the transcriptional comparison between the two strains under each condition. Under aerobic conditions, 100 genes were up-regulated and 186 genes down-regulated in the *nsrR* mutant compared to the WT strain. The mutation of *nsrR* had an even bigger effect anaerobically; with 270 genes up-regulated and 315 genes down-regulated. Twenty genes were found to have a higher expression level in the *nsrR* mutant under both aerobic and anaerobic conditions. Of these, eight have been found to be NsrR regulated and were shown to be induced in the absence of NsrR. Four of them have been associated with tellurite resistance and the other five are known to be involved in anaerobic nitrate respiration and NO detoxification (Table 17 and Figure 39). Of the 315 genes that showed down-regulation in the *nsrR* mutant compared to the WT strain anaerobically, 56 genes were found to be down-regulated under aerobic conditions in the $\Delta nsrR$ mutant. The transcriptional changes between the anaerobic and the aerobic growth of SL1344 WT will be first discussed to provide an overview of the genes that are important during anaerobic nitrate respiration. Afterwards the transcriptional differences between the WT strain and the *nsrR* mutant will be discussed for both growth conditions.

5.4.3.1 Transcriptional differences between anaerobic vs. aerobic growth of SL1344 WT

Genes have been categorized functionally according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) in combination with recent publications. Figure 35 and Figure 36 give an overview of the transcriptional changes of genes during the shift from aerobic to anaerobic growth in SL1344 WT and $\Delta nsrR$, respectively. Some of the functional categories that are most relevant to this study are now discussed in more detail for the WT strain. These include genes involved in nitrate metabolism, NO detoxification, oxidative stress, vitamin B12 biosynthesis and widely uncharacterised sRNA. A full list of the genes of these functional categories is given in Table 14 for the WT strain and Appendix E for $\Delta nsrR$. A detailed comparison between the WT strain and the *nsrR* mutant will be given in the next section and therefore will not be discussed any further at this point.

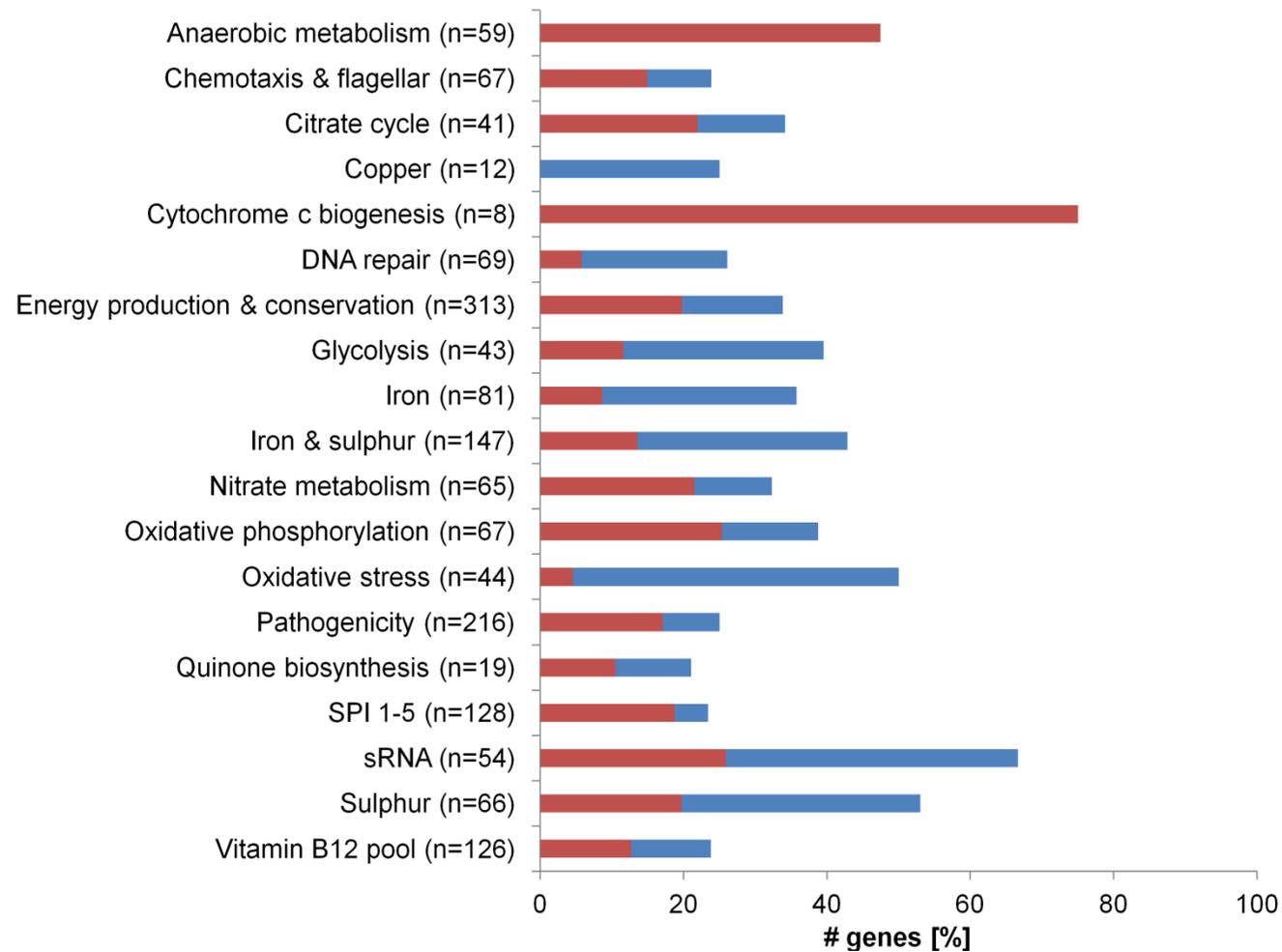


Figure 35 Relative percentage of genes up-/down-regulated in *S. Typhimurium* WT during anaerobiosis compared to aerobic growth.

Genes of interest were categorized according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) in combination with recent publications. Red bars = [%] of genes up-regulated during anaerobiosis; blue bars = [%] of genes down-regulated during anaerobiosis. n=number of genes. Cut-off threshold = 2-fold.

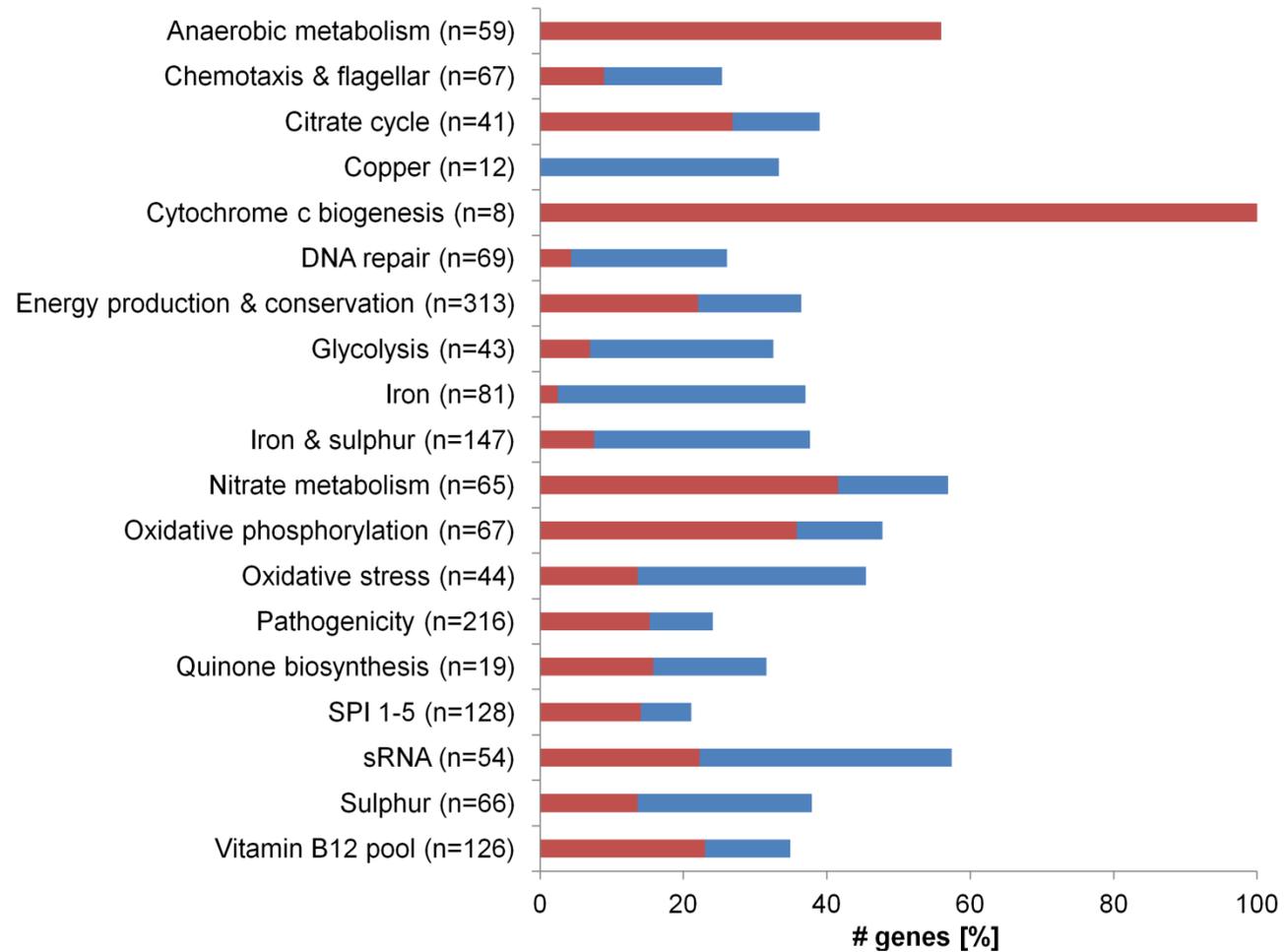


Figure 36 Relative percentage of genes up-/down-regulated in *S. Typhimurium* $\Delta nsrR$ during anaerobiosis compared to aerobic growth. Genes of interest were categorized according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) in combination with recent publications. Red bars = [%] of genes up-regulated during anaerobiosis; blue bars = [%] of genes down-regulated during anaerobiosis. n=number of genes. Cut-off threshold = 2-fold.

Nitrate metabolism

In agreement with the qRT-PCR data, genes of the *nap* and *nrf* operon were found to be up-regulated in the SL1344 WT microarray, reflecting the glycerol-limited growth conditions (Table 14). In addition, the nitrate assimilation operon *glnALG* was induced to positively regulate genes involved in the utilization of poor nitrogen sources (MacNeil et al., 1982). The gene encoding the iron-sulphur cluster repair di-iron protein, YtfE, was also up-regulated. This is not surprising as nitric oxide damages the [Fe-S] clusters that are contained in many proteins involved in anaerobic nitrate respiration, including the [Fe-S] clusters containing transcriptional regulators FNR, Fur and NsrR. These regulators were found to be down-regulated anaerobically in this study (Table 14). Another explanation for the up-regulation of *ytfE* is that *ytfE*, which has been previously shown to be NsrR regulated, becomes derepressed upon down-regulation of *nsrR* (Bodenmiller and Spiro, 2006, Filenko et al., 2007, Karlinsey et al., 2012). Surprisingly, two other NsrR repressed genes, *yoaG* and *ogt*, were found to be down-regulated as well. However, they are both further regulated by NarL (Squire et al., 2009, Lin et al., 2007). Thus, it is possible that *yoaG* and *ogt* are only active during nitrate-sufficient growth conditions, since activation by NarL requires high levels of nitrate or nitrite that are not found under the present conditions. Since the *narUZYWV* operon is active under carbon starvation and repressed by the reduced form of OxyR in the absence of exogenous H₂O₂ (Spector et al., 1999), it makes sense that *narZ* was found to be down-regulated anaerobically.

Oxidative stress

A few of the genes that are changing during anaerobiosis compared to aerobic growth have been ascribed functions related to oxidative stress management. *Salmonella* uses at least four global regulatory systems, SoxRS, OxyRS, FNR and ArcAB, to gain protection against oxidative stress that they encounter during their lifecycle (Calderon et al., 2011). SoxRS and OxyRS are involved in sensing and activating defence mechanisms against redox-cycling agents such as the superoxide anion (O₂^{•-}) and hydrogen peroxide (H₂O₂), respectively

(Zheng et al., 1999). Activated OxyR induces transcription of more than 20 genes that are required for oxidative stress defence including: three catalases (*katG*, *katE* and *katN*) and three peroxidases (*ahpC*, *tsaA* and *tpx*) that are involved in H₂O₂ breakdown and reduction of oxidized lipids; *dps* (involved in DNA protection); genes important for disulfide bond formation that are part of the thioredoxin (*trxA*, *trxB*, *trxC* and *dsbC*) and glutathione/glutaredoxin systems (*gshA*, *gshB*, *gorA*, *grxA*, *grxB* and *grxC*); and *fur* (encoding the Fur ferric ion uptake repressor Fur) (Calhoun and Kwon, 2011, Hebrard et al., 2009, Spector and Kenyon, 2012, Runkel et al., 2013, Bjur et al., 2006, Horst et al., 2010, Paget and Buttner, 2003). Next to its primary role of mediating a response to superoxide, SoxRS was also shown to be activated by NO by nitrosylation of the [2Fe-2S] clusters of the protein (Ding and Demple, 2000). The oxidised form of SoxR activates transcription of *soxS*, which in turn induces transcription of genes of the SoxRS regulon. These include the superoxide dismutases (*sodA*, *sodB* and *sodC*), *nfsA* (a NADPH-dependent nitroreductase to prevent O₂^{·-} formation), a glucose-6-phosphate dehydrogenase (*zwf*) and the endonuclease IV (*nfo*) for DNA repair (Daugherty et al., 2012, Runkel et al., 2013, Ding and Demple, 2000). In this study, most of the OxyR and SoxR regulated genes were down-regulated anaerobically (Table 14). This was not surprising since reactive oxygen species were not expected to form during anaerobic growth of glycerol-sufficient/nitrate-limited chemostat cultures. However, it was noticeable that *nmpC*, the gene expressing the outer membrane porin OmpD, was highly up-regulated. OmpD is the most abundant porin in *S. Typhimurium* and facilitates uptake of hydrogen peroxide, besides its involvement in antimicrobial peptide resistance (Calderon et al., 2011, Spector and Kenyon, 2012). Its expression is enhanced during anaerobiosis and it is repressed by ArcA (Santiviago et al., 2003, Calderon et al., 2011).

Table 14 Genes more than 2-fold higher/lower transcribed in *S. Typhimurium* WT anaerobically vs. aerobic growth conditions from G⁺/N⁻ chemostat cultures.

Category	Gene	Fold change	Function
Nitrate metabolism Up-regulated	<i>glnA</i>	4.42	glutamine synthetase
	<i>glnG</i>	2.33	nitrogen regulation protein NR(I)
	<i>glnL</i>	2.30	nitrogen regulation protein NR(II)
	<i>metR</i>	2.24	metE/metH regulator
	<i>napA</i>	2.98	nitrate reductase catalytic subunit
	<i>napB</i>	2.43	citrate reductase cytochrome c-type subunit
	<i>napC</i>	2.49	cytochrome c-type protein NapC
	<i>napG</i>	4.03	quinol dehydrogenase periplasmic component
	<i>napH</i>	2.04	quinol dehydrogenase membrane component
	<i>nirC</i>	2.74	nitrite transporter NirC
	<i>nrfA</i>	2.45	cytochrome c552
	<i>nrfC</i>	2.58	putative formate-dependent nitrite reductase
	<i>nrfG</i>	2.32	formate-dependent nitrite reductase complex subunit NrfG
	<i>ytfE</i>	2.24	iron-sulfur cluster repair di-iron protein
	Nitrate metabolism Down-regulated	<i>fnr</i>	2.22
<i>fur</i>		15.39	ferric uptake regulator
<i>narZ</i>		9.93	nitrate reductase 2 alpha subunit
<i>ogt</i>		10.33	O-6-alkylguanine-DNA:cysteine-protein methyltransferase
<i>STM1809</i>		7.64	putative cytoplasmic protein;GnsA/GnsB family
<i>yjeB</i>		4.02	transcriptional repressor NsrR
<i>yoaG</i>		3.48	putative cytoplasmic protein
Oxidative stress Up-regulated	<i>katG</i>	2.79	hydroperoxidase
	<i>nmpC</i>	62.11	putative outer membrane porin precursor; <i>ompD</i>
Oxidative stress Down-regulated	<i>arcA</i>	7.10	two-component response regulator
	<i>dksA</i>	5.76	DnaK transcriptional regulator DksA
	<i>dps</i>	24.83	DNA starvation/stationary phase protection protein Dps
	<i>dsbC</i>	2.36	thiol:disulfide interchange protein DsbC
	<i>fnr</i>	2.22	fumarate/nitrate reduction transcriptional regulator
	<i>fur</i>	15.39	ferric uptake regulator
	<i>grxB</i>	3.70	glutaredoxin 2
	<i>gshA</i>	2.09	glutamate--cysteine ligase
	<i>htpX</i>	2.07	heat shock protein HtpX
	<i>msrA</i>	2.48	methionine sulfoxide reductase A
	<i>rseA</i>	2.10	anti-RNA polymerase sigma factor SigE
	<i>sodA</i>	7.86	superoxide dismutase
	<i>sodC</i>	10.64	superoxide dismutase
	<i>tpx</i>	4.23	thiol peroxidase
	<i>trxA</i>	3.27	thioredoxin
	<i>trxB</i>	5.77	thioredoxin reductase
	<i>trxC</i>	3.60	thioredoxin 2
	<i>tsaA</i>	2.03	putative thiol-alkyl hydroperoxide reductase
	<i>ydgQ</i>	2.19	electron transport complex RsxE subunit
	<i>zwf</i>	2.31	glucose-6-phosphate 1-dehydrogenase

Table 14 cont.

Category	Gene	Fold change	Function
sRNA Up-regulated	<i>cyaR</i>	52.91	Unknown
	<i>glmY</i>	2.04	Unknown
	<i>glnL</i>	2.30	nitrogen regulation protein NR(II)
	<i>isrA</i>	8.77	Unknown
	<i>isrH_1_2</i>	3.85	Unknown
	<i>micF</i>	2.46	Unknown
	<i>orf7</i>	3.79	Unknown
	<i>PSLT065</i>	2.16	Unknown
	<i>PSLT071</i>	2.80	Unknown
	<i>rseX</i>	2.22	Unknown
	<i>rydC</i>	2.26	Unknown
	<i>ryeC</i>	3.45	Unknown
	<i>srgA</i>	2.05	Unknown
	<i>t44</i>	2.13	Unknown
sRNA Down-regulated	<i>csrC</i>	7.98	putative cytoplasmic protein
	<i>dsrA</i>	25.64	Unknown
	<i>isrI</i>	3.17	Unknown
	<i>isrJ</i>	4.08	Unknown
	<i>isrK</i>	2.17	Unknown
	<i>isrN</i>	2.00	Unknown
	<i>isrP</i>	3.66	Unknown
	<i>istR</i>	4.71	Unknown
	<i>oxyS</i>	15.55	Unknown
	<i>PSLT026</i>	3.59	putative periplasmic protein
	<i>rprA</i>	2.81	Unknown
	<i>rybA</i>	2.12	Unknown
	<i>ryeB</i>	46.69	putative cytoplasmic protein
	<i>ryhB-2</i>	23.54	Unknown
	<i>sgrS</i>	2.03	Unknown
	<i>spf</i>	486.90	Unknown
	<i>spvD</i>	2.26	Unknown
	<i>sraA</i>	3.42	Unknown
	<i>sraG</i>	2.05	Unknown
	<i>sroB</i>	4.45	Unknown
<i>sroC</i>	2.53	Unknown	
<i>tp2</i>	9.39	Unknown	
Vitamin B12 Up-regulated	<i>dcm</i>	2.04	DNA cytosine methylase
	<i>eutS</i>	2.83	putative carboxysome structural protein
	<i>metB</i>	3.36	cystathionine gamma-synthase
	<i>metF</i>	4.55	5,10-methylenetetrahydrofolate reductase
	<i>metH</i>	2.81	B12-dependent methionine synthase
	<i>metK</i>	2.74	S-adenosylmethionine synthetase
	<i>pduA</i>	2.05	polyhedral body protein
	<i>pduD</i>	2.05	propanediol dehydratase medium subunit
	<i>pduE</i>	2.18	propanediol dehydratase small subunit
	<i>pduF</i>	2.03	propanediol diffusion facilitator; Unknown
	<i>pduK</i>	2.13	polyhedral body protein
	<i>pduP</i>	2.08	CoA-dependent propionaldehyde dehydrogenase
	<i>pduQ</i>	2.57	propanol dehydrogenase
	<i>pduS</i>	2.02	polyhedral body protein
	<i>speD</i>	3.83	S-adenosylmethionine decarboxylase
<i>STM3073</i>	2.06	putative cobalt ABC transporter	

Table 14 cont.

Category	Gene	Fold change	Function
Vitamin B12 Down-regulated	<i>aspC</i>	4.78	aromatic amino acid aminotransferase
	<i>cbiO</i>	2.31	cobalt transporter ATP-binding subunit
	<i>cyoE</i>	2.19	protoheme IX farnesyltransferase
	<i>cysE</i>	2.50	serine acetyltransferase
	<i>cysK</i>	2.38	cysteine synthase A
	<i>cysM</i>	2.02	cysteine synthase B
	<i>exbD</i>	2.82	biopolymer transport protein ExbD
	<i>ftnB</i>	9.66	ferritin-like protein
	<i>hemA</i>	3.08	glutamyl-tRNA reductase
	<i>hemL</i>	3.76	glutamate-1-semialdehyde aminotransferase
	<i>luxS</i>	8.09	S-ribosylhomocysteinase
	<i>sdaA</i>	4.38	L-serine deaminase I/L-threonine deaminase I
	<i>yedO</i>	3.58	D-cysteine desulfhydrase

Since *arcA* was down-regulated during anaerobiosis in the chemostat cultures (Table 14) *ompD* became derepressed, resulting in the high expression level detected. Additionally, the gene encoding the bi-functional catalase/peroxidase KatG was up-regulated upon anaerobiosis (Table 14), but the reason for this remains to be elucidated.

Vitamin B12

Some of the genes that were found to be more than 2-fold up-/down regulated during anaerobiosis are associated with the vitamin B12 metabolism (Table 14). Vitamin B12, also known as cobalamin, is chemically the most complex vitamin and is known to be produced by only certain bacteria and archaea (Roth et al., 1996). Since many animals, including humans, require vitamin B12 but are unable to synthesize it, they have to obtain it via their diet. *Salmonella* spp. synthesize vitamin B12 only anaerobically and require the *cob* operon, which includes the *cob* and *cbi* genes, to produce the vitamin B12 precursor adenosyl cobalamin (Jeter et al., 1984, Lawhon et al., 2003, Roth et al., 1996). *Salmonella* use vitamin B12 for at least three reactions; degradation of ethanolamine, 1,2-propanediol and for the synthesis of methionine. Ethanolamine and 1,2-propanediol serve as carbon and energy sources and require, next to the expression of the genes of the *pdu* and *eut* operons, tetrathionate as a terminal electron acceptor for anaerobic utilization (Price-Carter et al., 2001). Apart from *Salmonella*, only two other pathogenic bacterial species, *Clostridium perfringens* and *Listeria monocytogenes*, are able to use

both 1,2-propanediol and ethanolamine as a sole carbon source (Srikumar and Fuchs, 2011). *Salmonella* possesses two methionine synthases; the vitamin B12-dependent MetH and MetE, which can catalyze the same reaction without vitamin B12 (Roth et al., 1996). Recently, in *Paracoccus* a link between N₂O, the vitamin B12 pool and B12 riboswitches has been made (Sullivan et al., 2013). N₂O was shown to exhibit a cytotoxic effect by binding to and inactivating the vitamin B12 pool and MetH, which resulted in the up-regulation of *metE* (Sullivan et al., 2013, Drummond and Matthews, 1994). The same group demonstrated that the cytotoxic effect can be relieved by the addition of exogenous vitamin B12 or L-methionine. These findings even have a huge impact on humans, since B12-deficiency and hyperhomocysteinemia have been previously reported as a consequence of N₂O-based anaesthetics (Sullivan et al., 2013, Badner et al., 2000). In this study, genes of the *pdu* operon as well as *metH* and *eutS* were up-regulated, while some others involved in carbon storage (*csrA*) or cobalt (*cbiO*) and vitamin B12 (*exbD*) transport were down-regulated anaerobically (Table 14). The increased expression of *metH* might be indicative for an intact vitamin B12 pool, as it is used preferentially when B12 is available (Roth et al., 1996). This fits to the relatively low N₂O levels that accumulated only transiently in the WT chemostat culture (Figure 32). Alternatively, it could also be possible that *Salmonella* tried to make more of MetH to counter the effect of N₂O.

sRNA

Small non-coding RNAs (sRNAs) are a ubiquitous class of regulatory elements that are involved in posttranscriptional gene regulation processes including nutrient availability, anaerobic growth, iron homeostasis and stress responses such as oxidative, envelope and osmotic stress (Hebrard et al., 2012, Kroger et al., 2012, Papenfort and Vogel, 2010). Many of them have multiple target genes and were shown to accumulate predominantly during the stationary phase of growth (Frohlich et al., 2012). One of the sRNAs that were up-regulated during anaerobiosis in the SL1344 WT chemostat culture was *cyaR*, a conserved CRP-dependent riboregulator of OmpX synthesis (Table 14). OmpX is repressed by CyaR (former RyeE) (Papenfort et al., 2008) and was found to be

down-regulated (18-fold) in this study. Other up-regulated sRNAs include *glmY* (important for cell wall biosynthesis), the previously mentioned nitrogen regulation protein *glnL* and a pathogenicity island associated *isrA* gene with unknown function (Hebrard et al., 2012, Kroger et al., 2012, Gopel et al., 2011). Slightly more sRNAs were down-regulated anaerobically (Table 14). *spf*, also known as spot 42, which had an aerobic relative expression level (REL) of 200 was down-regulated anaerobically by approximately 500-fold. Spot 42, an important regulator in the carbohydrate metabolism, is activated by glucose and inhibited by the cAMP-CRP complex (Gorke and Vogel, 2008). The same group showed that it selectively inhibits the synthesis of the galactokinase GalK but not the GalE and GalT proteins. Spot 42 concentrations were found to be significantly lower during growth with a non-glucose carbon source (Hansen et al., 2012), which was glycerol in this study. Another highly down-regulated gene was *dsrA*, which is an activator of the stress responsive sigma factor RpoS (Frohlich et al., 2012). As a consequence, *rpoS* was found to be down-regulated 8-fold, which in turn resulted in the down-regulation of *ryeB* (Table 14), a conserved RpoS-dependent small RNA that is one of the most abundant stationary phase-specific sRNAs in *E. coli* and which controls the synthesis of OmpD (Frohlich et al., 2012). Furthermore, the oxidative stress induced sRNAs, *oxyS* and *ryhB*, were 15- and 23-fold down-regulated anaerobically (Table 14) (Altuvia et al., 1997, Calderon et al., 2014). In addition to oxidative stress defence, *ryhB* is also involved in iron homeostasis (Calderon et al., 2014).

5.4.3.2 Transcriptional differences between SL1344 Δ *nsrR* vs. WT

Overall, it was noticeable that the *nsrR* mutant possesses more genes that exceed the 2-fold cut-off threshold compared to the WT; most of which are involved in nitrate metabolism and NO detoxification. Furthermore, it became apparent that the genes that were up-regulated anaerobically in both strains were even higher induced in the *nsrR* mutant (Table 14 and Appendix E). An overview of the transcriptional differences between Δ *nsrR* and the WT strain is given in Figure 37 (aerobic comparison) and Figure 38 (anaerobic comparison).

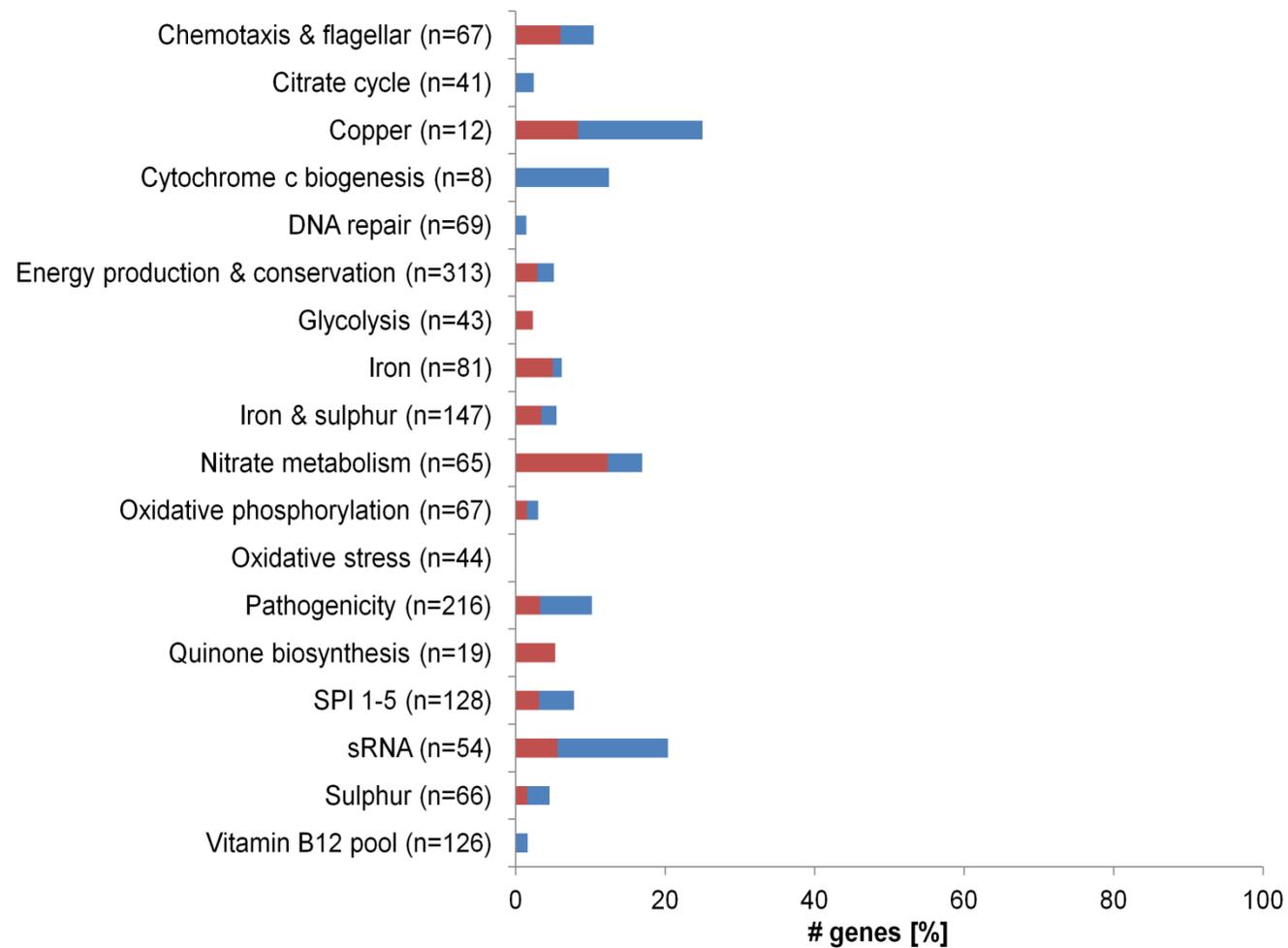


Figure 37 Relative percentage of genes that were higher/lower transcribed in *S. Typhimurium* $\Delta nsrR$ vs. WT during aerobic growth.

Genes of interest were categorized according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) in combination with recent publications. Red bars = [%] of genes that were higher expressed in $\Delta nsrR$; blue bars = [%] of genes that were lower expressed in $\Delta nsrR$. n=number of genes. Cut-off threshold = 2-fold.

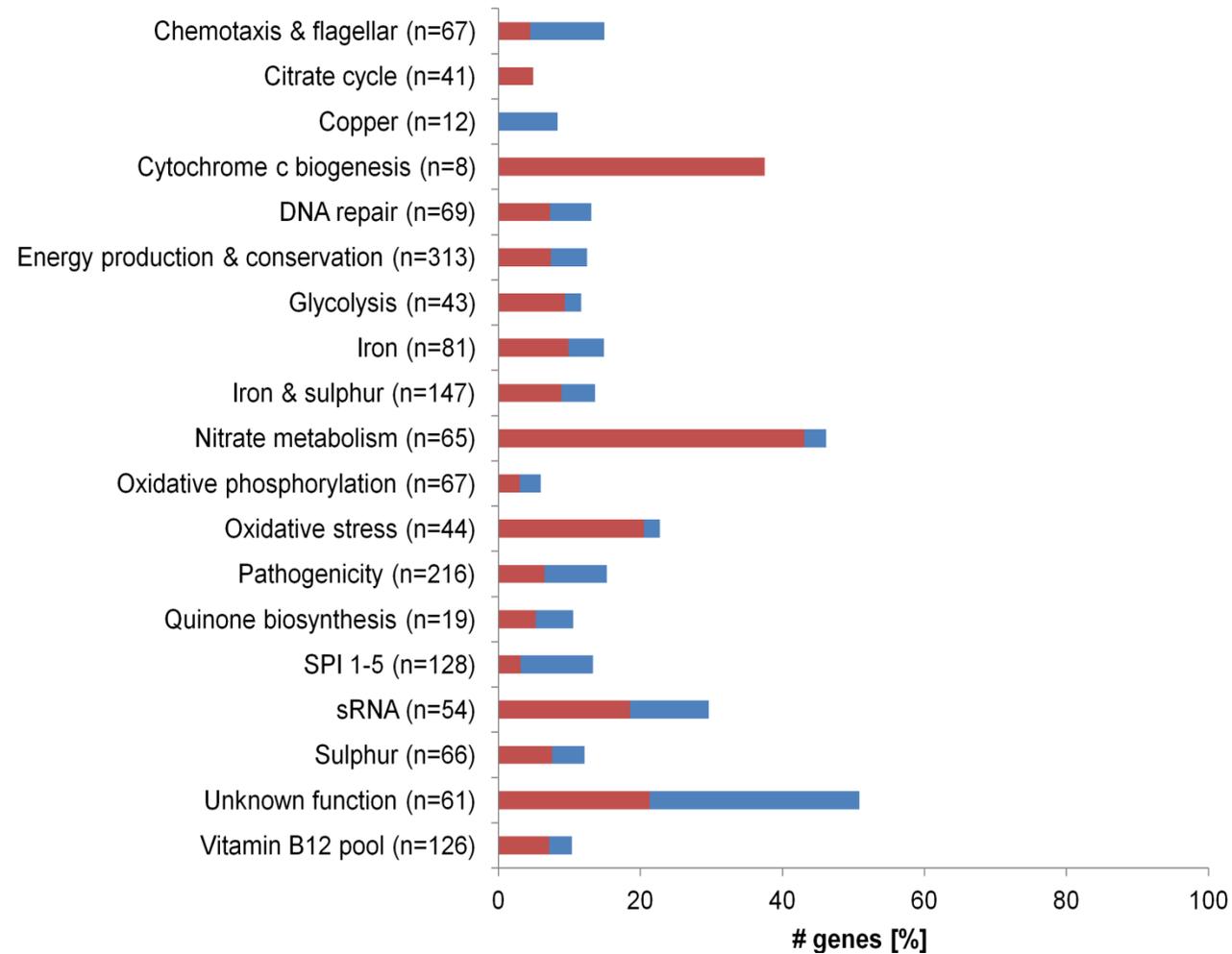


Figure 38 Relative percentage of genes that were higher/lower transcribed in *S. Typhimurium* $\Delta nsrR$ vs. WT during anaerobic growth. Genes of interest were categorized according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) in combination with recent publications. Red bars = [%] of genes that were higher expressed in $\Delta nsrR$; blue bars = [%] of genes that were lower expressed in $\Delta nsrR$. n=number of genes. Cut-off threshold = 2-fold.

A list of genes, categorized in functional groups that are relevant to this study, is given in Table 15 (aerobic comparison) and Table 16 (anaerobic comparison).

Nitrate metabolism and nitrosative stress

As mentioned above, a number of genes exhibited higher expression in the absence of NsrR; many of which were found to be associated with the nitrate metabolism and which are repressed by NsrR. As an example, the whole *nap* operon was strongly up-regulated in $\Delta nsrR$ (Appendix E) as well as most of the *nrf* operon, in comparison to the WT strain which had only a few genes of each operon induced (Table 14). In addition to the two NsrR-regulated operons (*nrf* and *nap*) the *nirBCD* and the *narGHJI* operons, which were only induced in the *nsrR* mutant, were also found to be more highly expressed (Table 16). This agrees with the qRT-PCR data (Figure 31 and Figure 34). Furthermore, all genes that have previously been shown to be repressed by NsrR (Table 13) were found to be higher expressed in $\Delta nsrR$ under both aerobic and anaerobic conditions. The transcriptional differences of these genes (fold-change $\Delta nsrR$ vs. WT) are presented in Table 17 and Figure 39. *tehB* was excluded from this, because although it was 2.5-fold higher transcribed aerobically (Table 15), it missed the cut-off threshold by having only a 1.9-fold difference under anaerobic conditions. In order to analyze the results thoroughly and to make a meaningful comparison between the two strains, the relative expression levels of each gene and their transcriptional trend (up- or down-regulation) always have to be considered. Although, all of the genes of Table 13 were higher expressed in $\Delta nsrR$ compared to the WT strain, only *hmpA* and the *hcp-hcr* operon were up-regulated in the *nsrR* mutant when comparing the aerobic with the anaerobic phase. The operon that was the most up-regulated in $\Delta nsrR$ (-O₂ vs. +O₂) was the *hcp-hcr* operon (Appendix E), which was shown before to be maximally induced during anaerobic conditions in the presence of nitrite (Chismon et al., 2010). However, the aerobic relative expression levels of *hcp-hcr* were 3 and 1.6 respectively, suggesting that the derepression by NsrR alone could not have caused the high expression.

Table 15 Genes more than 2-fold higher/lower transcribed in *S. Typhimurium* Δ *nsrR* vs. WT aerobically under G^+/N^- conditions.

Category	Gene	Fold change	Function
Nitrate metabolism higher-transcribed	<i>hcp</i>	2.87	unclear
	<i>hcr</i>	3.25	Hcp oxidoreductase, NADH-dependent
	<i>hmpA</i>	22.47	nitric oxide dioxygenase
	<i>STM1808</i>	27.46	putative cytoplasmic protein
	<i>tehB</i>	2.47	tellurite resistance protein TehB
	<i>yeaR</i>	8.86	putative cytoplasmic protein
	<i>ygbA</i>	10.17	hypothetical protein
	<i>yoaG</i>	9.28	putative cytoplasmic protein
	<i>ytfE</i>	120.50	iron-sulfur cluster repair di-iron protein
Nitrate metabolism lower-transcribed	<i>napF</i>	2.40	ferredoxin-type protein
	<i>nrfB</i>	2.21	Unknown
	<i>nrfC</i>	2.37	putative formate-dependent nitrite reductase
Pathogenicity higher-transcribed	<i>fliC</i>	4.78	flagellin
	<i>istR</i>	2.49	Unknown
	<i>rmbA</i>	2.59	putative cytoplasmic protein
	<i>sicP</i>	2.04	secretion chaparone
	<i>sifA</i>	2.25	secreted effector protein
	<i>ssrA</i>	2.31	sensor kinase
	<i>STM4257</i>	3.07	hypothetical protein
Pathogenicity lower-transcribed	<i>fimC</i>	2.10	periplasmic chaperone
	<i>fimI</i>	2.25	fimbrial protein
	<i>fimY</i>	2.24	putative regulatory protein
	<i>fliB</i>	4.24	flagellin
	<i>hilC</i>	2.84	invasion regulatory protein
	<i>iagB</i>	2.25	invasion protein precursor
	<i>oafA</i>	2.12	O-antigen acetylase
	<i>orf408</i>	2.23	putative regulatory protein
	<i>orfX</i>	2.16	putative cytoplasmic protein
	<i>sopE2</i>	2.14	type III-secreted effector protein
	<i>spvR</i>	2.19	Unknown
	<i>ssaT</i>	2.48	type III secretion system apparatus protein
	<i>STM1870</i>	3.86	Unknown;RecE-like protein
	<i>STM2231</i>	2.01	virulence protein
<i>STM2914</i>	2.60	putative nucleoside-diphosphate-sugar epimerase	
sRNA higher-transcribed	<i>isrA</i>	17.62	Unknown
	<i>istR</i>	2.49	Unknown
	<i>orf7</i>	4.19	Unknown
sRNA lower-transcribed	<i>cyaR</i>	2.37	Unknown
	<i>isrG</i>	3.30	Unknown
	<i>isrK</i>	2.33	Unknown
	<i>isrP</i>	2.72	Unknown
	<i>PSLT065</i>	2.17	Unknown
	<i>rydB</i>	2.09	Unknown
	<i>spvR</i>	2.19	Unknown
	<i>tpke11</i>	11.55	Unknown
Vitamin B12 lower-transcribed	<i>cbiN</i>	2.39	cobalt transport protein CbiN
	<i>pduK</i>	3.30	polyhedral body protein

Activation by NarXL and NarQP could have further contributed to the high expression levels of *hcp-hcr*. In contrast to *hcp-hcr*, *hmpA* was up-regulated less than 2-fold in the *nsrR* mutant anaerobically compared to the aerobic expression level and was therefore not included in Appendix E. However, the reason for this was found when comparing the *hmpA* expression levels of the *nsrR* mutant with that of the WT strain (Table 17 and Figure 39). Since NsrR is the principal regulator of *hmpA* during nitrosative stress (Bang et al., 2006), *hmpA* becomes completely deregulated in its absence, resulting in extremely high expression levels of *hmpA* even during the aerobic phase (Table 15, Table 17 and Figure 39). The aerobic REL of *hmpA* in $\Delta nsrR$ was 40 while that of the WT strain was only 2. The high transcription of genes known to be involved in NO production (*narG*) and detoxification (*hmpA* and potentially *hcp-hcr*) explain the high N₂O levels seen in the $\Delta nsrR$ chemostat culture (Figure 33).

Two other genes that seemed to be deregulated in the *nsrR* mutant are *ytfE* and *STM1808*. Both were highly expressed aerobically (REL = 108 and 62 respectively) but slightly down-regulated during anaerobic growth (REL = 46 and 38 respectively). Nonetheless, they were much higher transcribed under aerobic as well as anaerobic conditions in comparison to the WT strain (Table 15-17). In addition to being derepressed in the absence of NsrR, *ytfE* was potentially further derepressed by FNR and Fur, which were both down-regulated anaerobically and predicted to regulate *ytfE* (Justino et al., 2006). However, no obvious Fnr or Fur binding sites were found. Although *ytfE* and *STM1808* were down-regulated, both of their REL during anaerobiosis were relatively high, indicating that they might play an important role during anaerobic nitrate respiration. This is no surprise for YtfE, since it is involved in the [Fe-S] cluster repair of NO damaged proteins. The three remaining genes (*yeaR-yoaG* and *ygbA*) were less highly expressed in comparison to the other NsrR-regulated genes. Their aerobic REL was 20, 23 and 8 respectively and they were less than 10-fold higher transcribed in the *nsrR* mutant compared to the WT strain (Table 17).

Table 16 Genes more than 2-fold higher and lower transcribed in *S. Typhimurium ΔnsrR* vs. WT anaerobically under G⁺/N⁻ conditions.

Category	Gene	Fold change	Function
Cytochrome c Biogenesis higher-transcribed	<i>ccmA</i>	2.65	cytochrome c biogenesis protein CcmA
	<i>ccmC</i>	3.50	heme exporter protein
	<i>ccmE</i>	2.23	cytochrome c-type biogenesis protein CcmE
Iron & sulphur metabolism higher-transcribed	<i>bfr</i>	2.80	bacterioferritin
	<i>cysJ</i>	2.01	sulfite reductase subunit alpha
	<i>dps</i>	5.54	DNA starvation/stationary phase protection protein
	<i>exbD</i>	2.02	biopolymer transport protein ExbD
	<i>fur</i>	3.57	ferric uptake regulator
	<i>glpE</i>	2.14	thiosulfate sulfurtransferase
	<i>gpmA</i>	2.46	phosphoglyceromutase
	<i>moaC</i>	2.13	molybdenum cofactor biosynthesis protein C
	<i>moaE</i>	2.13	molybdopterin synthase large subunit
	<i>sbp</i>	2.27	sulfate transporter subunit
	<i>sufA</i>	3.38	iron-sulfur cluster assembly scaffold protein
	<i>ydhD</i>	2.75	hypothetical protein
	<i>ytfE</i>	23.10	iron-sulfur cluster repair di-iron protein
	Iron & sulphur metabolism lower-transcribed	<i>dmsB</i>	2.46
<i>entC</i>		2.40	isochorismate synthase
<i>entF</i>		2.90	enterobactin synthase subunit F
<i>iroE</i>		2.45	putative hydrolase
<i>moeA</i>		4.22	molybdopterin biosynthesis protein MoeA
<i>phsB</i>		3.70	thiosulfate reductase electron transport protein
<i>ytfK</i>		2.98	Protein of unknown function
Nitrate metabolism higher-transcribed	<i>fdnG</i>	2.54	formate dehydrogenase-N alpha subunit
	<i>fdnI</i>	2.66	formate dehydrogenase-N subunit gamma
	<i>fur</i>	3.57	ferric uptake regulator
	<i>glnL</i>	2.22	Unknown;nitrogen regulation protein NR(II)
	<i>hcp</i>	21.89	unclear
	<i>hcr</i>	40.71	Hcp oxidoreductase, NADH-dependent
	<i>hmpA</i>	14.67	nitric oxide dioxygenase
	<i>napA</i>	5.30	nitrate reductase catalytic subunit
	<i>napB</i>	3.32	citrate reductase cytochrome c-type subunit
	<i>napC</i>	5.11	cytochrome c-type protein NapC
	<i>napD</i>	2.45	assembly protein for periplasmic nitrate reductase
	<i>napG</i>	4.33	quinol dehydrogenase periplasmic component
	<i>napH</i>	3.37	quinol dehydrogenase membrane component
	<i>narG</i>	3.49	nitrate reductase 1 alpha subunit
	<i>narH</i>	5.98	nitrate reductase 1 beta subunit
	<i>narI</i>	3.64	nitrate reductase 1 gamma subunit
	<i>narJ</i>	8.15	nitrate reductase 1 delta subunit
<i>narK</i>	2.34	nitrite extrusion protein	

Table 16 cont.

Category	Gene	Fold change	Function
	<i>nirB</i>	3.82	nitrite reductase large subunit
	<i>nirD</i>	3.83	nitrite reductase small subunit
	<i>nrfC</i>	2.65	putative formate-dependent nitrite reductase
	<i>ogt</i>	2.03	O-6-alkylguanine-DNA:cysteine-protein methyltransferase
	<i>STM1808</i>	14.48	putative cytoplasmic protein
	<i>STM1809</i>	2.10	putative cytoplasmic protein
	<i>yeaR</i>	4.69	putative cytoplasmic protein
	<i>ygbA</i>	3.50	hypothetical protein
	<i>yoaG</i>	8.98	putative cytoplasmic protein
	<i>ytfE</i>	23.10	iron-sulfur cluster repair di-iron protein
Nitrate metabolism lower-transcribed	<i>narV</i>	2.18	nitrate reductase 2 gamma subunit
	<i>soxR</i>	2.08	redox-sensing transcriptional activator
Oxidative stress higher-transcribed	<i>ahpC</i>	12.93	alkyl hydroperoxide reductase subunit C
	<i>ahpF</i>	5.21	alkyl hydroperoxide reductase F52a subunit
	<i>dps</i>	5.54	DNA starvation/stationary phase protection protein
	<i>fur</i>	3.57	ferric uptake regulator
	<i>grxA</i>	4.78	glutaredoxin 1
	<i>msrA</i>	2.15	methionine sulfoxide reductase A
	<i>trxB</i>	3.90	thioredoxin reductase
	<i>trxC</i>	6.20	thioredoxin 2
	<i>tsaA</i>	2.99	putative thiol-alkyl hydroperoxide reductase
Oxidative stress lower-transcribed	<i>soxR</i>	2.08	redox-sensing transcriptional activator
sRNA higher-transcribed	<i>csrC</i>	3.75	putative cytoplasmic protein
	<i>glmZ</i>	2.72	Unknown
	<i>glnL</i>	2.22	Unknown;nitrogen regulation protein NR(II)
	<i>invR</i>	2.23	Unknown
	<i>istR</i>	2.34	Unknown
	<i>orf6</i>	2.08	Unknown
	<i>oxyS</i>	4.70	Unknown
	<i>PSLT043</i>	2.11	type II secretion system protein
	<i>spf</i>	26.33	Unknown
	<i>spvC</i>	2.59	Unknown
sRNA lower-transcribed	<i>cyaR</i>	22.37	Unknown
	<i>isrH_1_2</i>	3.41	Unknown
	<i>pefB</i>	2.05	Unknown
	<i>PSLT065</i>	2.13	Unknown
	<i>rygC</i>	2.73	Unknown
	<i>srgA</i>	2.10	Unknown
Functional unknowns higher-transcribed	<i>SL2583</i>	2.90	Unknown
	<i>SL2585</i>	2.94	Unknown
	<i>SL2587</i>	4.83	Unknown
	<i>SL2588</i>	3.09	Unknown
	<i>SL2589</i>	2.97	Unknown
	<i>SL4256</i>	2.37	Unknown

Table 16 cont.

Category	Gene	Fold change	Function
	<i>STM0083</i>	2.42	Unknown
	<i>STM0436A</i>	2.09	Unknown; similar to IS903 transposase
	<i>STM2010</i>	3.77	Unknown
	<i>STM2880</i>	2.25	Unknown
	<i>STM3520</i>	2.16	Unknown; pseudo gene; tRNA
	<i>STM4030</i>	2.65	Unknown
	<i>STM3797A</i>	3.58	unknown; ribosomal slippage
Functional unknowns lower-transcribed	<i>hutU</i>	3.23	Unknown
	<i>SL1584</i>	2.66	Unknown
	<i>SL1954</i>	4.65	Unknown
	<i>SL1972</i>	12.56	Unknown
	<i>SL1974</i>	2.04	Unknown
	<i>SL2634</i>	3.24	Unknown
	<i>SL2637</i>	2.26	Unknown
	<i>SL2673</i>	3.28	Unknown;DNA invertase-like protein
	<i>SL2704</i>	2.82	Unknown;hypothetical protein
	<i>SL2719</i>	2.73	Unknown
	<i>SL4383</i>	7.04	Unknown
	<i>STM1861</i>	7.63	Unknown
	<i>STM2507</i>	2.53	Unknown
	<i>STM2682</i>	4.48	Unknown
	<i>STM2714</i>	2.57	Unknown;lysis-like protein
	<i>STM3035</i>	2.51	Unknown;putative endonuclease
	<i>STM4111</i>	2.92	Unknown
	<i>ytfK</i>	2.98	Unknown
Vitamin B12 higher-transcribed	<i>csrA</i>	2.98	carbon storage regulator
	<i>exbD</i>	2.02	biopolymer transport protein ExbD
	<i>luxS</i>	2.03	S-ribosylhomocysteinase
	<i>pduC</i>	2.54	propanediol dehydratase large subunit
	<i>pduE</i>	2.81	propanediol dehydratase small subunit
	<i>pduG</i>	2.17	propanediol dehydratase reactivation protein
	<i>pduH</i>	5.75	propanediol dehydratase reactivation protein
	<i>pudB</i>	2.37	polyhedral body protein
	<i>sdaA</i>	2.22	L-serine deaminase I/L-threonine deaminase I
Vitamin B12 lower-transcribed	<i>cobC</i>	2.62	alpha ribazole-5'-P phosphatase
	<i>metH</i>	2.28	B12-dependent methionine synthase
	<i>pduK</i>	3.15	polyhedral body protein
	<i>speD</i>	3.64	S-adenosylmethionine decarboxylase

Table 17 List of genes that were more than 2-fold higher transcribed in *S. Typhimurium* *AnsrR* vs. WT aerobically as well as anaerobically under G⁺/N⁻ conditions.

Category	Gene	Fold change (aerobic)	Fold change (anaerobic)	Function
Nitrate Metabolism	<i>hcp</i>	2.87	21.89	unclear
	<i>hcr</i>	3.25	40.71	Hcp oxidoreductase, NADH-dependent
	<i>hmpA</i>	22.47	14.67	nitric oxide dioxygenase
	<i>STM1808</i>	27.46	14.48	putative cytoplasmic protein
	<i>yeaR</i>	8.86	4.69	putative cytoplasmic protein
	<i>ygbA</i>	10.17	3.50	hypothetical protein
	<i>yoaG</i>	9.28	8.98	putative cytoplasmic protein
				iron-sulfur cluster repair di-iron protein
		<i>ytfE</i>	120.50	23.10

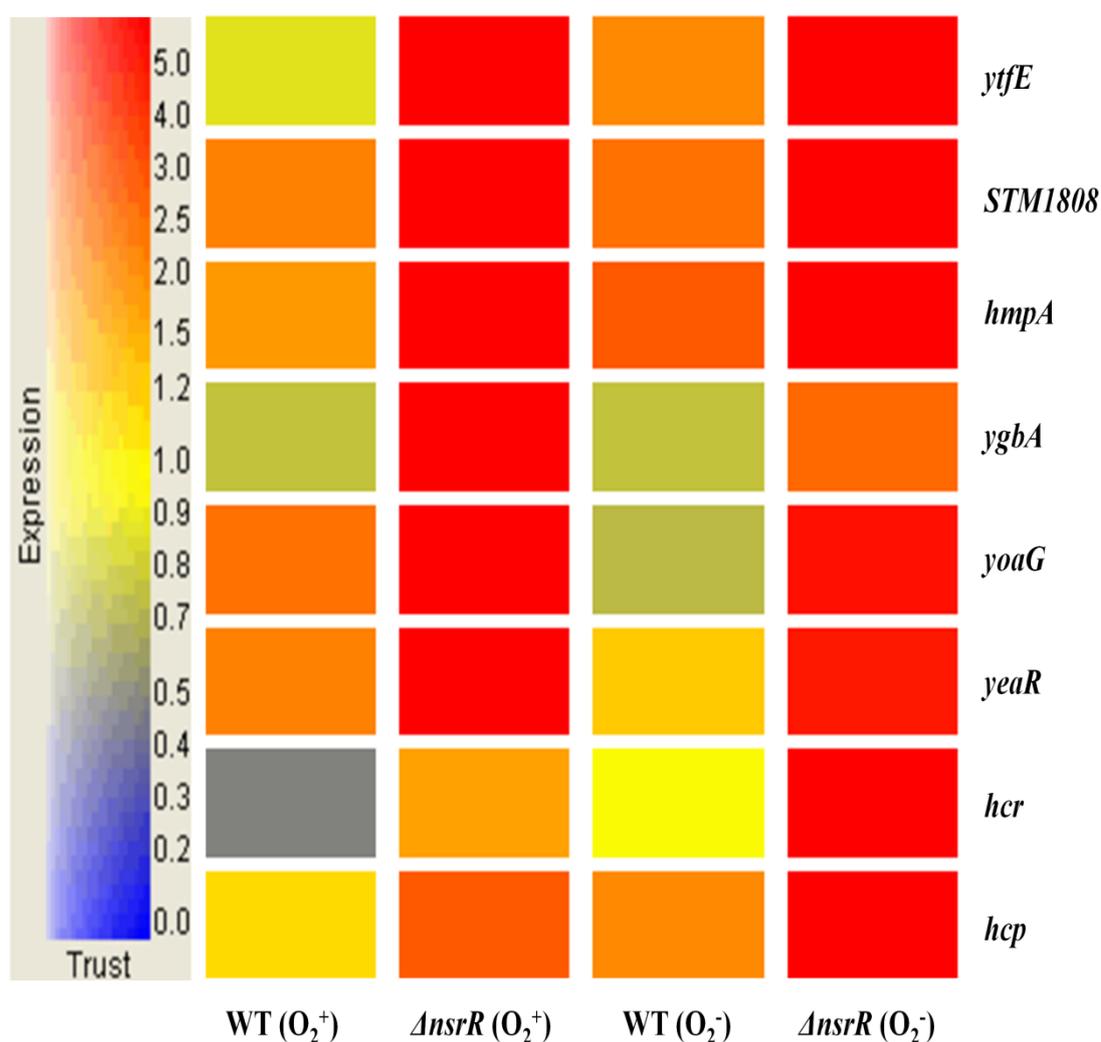


Figure 39 Heat map representing the different expression levels of selected genes of *S. Typhimurium* WT and *nsrR* mutant chemostat cultures during aerobic and anaerobic growth.

The chemostat was run under glycerol-sufficient (20 mM) and nitrate limited (5 mM) conditions. The genes are involved in anaerobic nitrate respiration.

Iron metabolism

As mentioned in different sections throughout this thesis, iron homeostasis must be tightly regulated in order to avoid cytotoxic effects of free iron (e.g. iron-catalyzed Fenton chemistry) and to maintain a sufficient amount of intracellular iron needed for a number of physiological processes including cellular respiration, DNA replication and repair or regulation of gene expression (Spector and Kenyon, 2012). Furthermore, iron serves as an important co-factor in proteins in the form of [Fe-S] clusters, which play a key role in anaerobic nitrate respiration in *Salmonella*. In this study, genes encoding the iron storage protein bacterioferritin (*bfr*) and Dps (*dps*) were higher transcribed in the *nsrR* mutant anaerobically compared to the WT strain, together with the transporter protein ExbD (*exbD*) (Table 16). These proteins are responsible for the binding and storage of excess intracellular iron (Velayudhan et al., 2007). However, they were down-regulated anaerobically in comparison to the aerobic expression level. The same was true for the gene encoding the [Fe-S] cluster assembly protein SufA (*sufA*) and the previously mentioned *ytfE* and *fur*. The expression pattern of the siderophores *entC* and *entF* were opposite to *ytfE*, *fur* and *sufA*; i.e. they were lower expressed anaerobically in $\Delta nsrR$ compared to the WT strain but they were found to be up-regulated under anaerobic conditions as opposed to aerobic conditions. Taken together, it seems that higher NO levels (based on the higher N₂O measured) in the mutant caused damage to the [Fe-S] clusters, which needed repairment by YtfE.

Oxidative stress

It was noticeable that genes involved in oxidative stress resistance were only found to be higher expressed in the *nsrR* mutant compared to the WT strain under anaerobic conditions (Figure 37 and Figure 38; Table 16). One of the genes that was up-regulated in the *nsrR* mutant during anaerobic vs. aerobic growth was *soxS*, which is part of the SoxRS regulon and important for protection against superoxide anions (Appendix E). However, as it was only 1.7-fold higher expressed in $\Delta nsrR$ compared to the WT strain, it was not listed in Table 16. The same is true for *sodB*, which was 1.9-fold higher expressed

anaerobically ($\Delta nsrR$ vs. WT). The gene encoding the aerobic superoxide dismutase SodA was 1.5-fold higher expressed aerobically in $\Delta nsrR$ compared to the WT strain. Furthermore, the two alkyl hydroperoxide reductase encoding genes, *ahpC* and *ahpF*, were 13 and 5-fold higher expressed in the *nsrR* mutant compared to the WT strain under anaerobic conditions (Table 16). Overall, this suggests that the *nsrR* mutant was exposed to higher levels of oxidative stress, which were presumably caused by an overexpressed *hmpA*. The theory is that the superoxide anions, produced aerobically by an overexpressed *hmpA*, became converted to H₂O₂ by the SODs, which in turn forms the highly reactive peroxyxynitrite upon exposure to NO. As a consequence, a lower growth rate was detected in the $\Delta nsrR$ chemostat culture (Figure 33). This is supported by another study, which showed that an *nsrR* mutation enhances the sensitivity of *Salmonella* to oxidative stress (Gilberthorpe et al., 2007).

Vitamin B12

In section 5.4.3.1 we have established that NO can interfere with the vitamin B12 pool. Since the *nsrR* mutant produced much more N₂O and therefore also NO, one would expect to see the genes of the vitamin B12-independent pathway to be higher transcribed. However, this was not the case. Mostly the *pdu* genes were higher transcribed anaerobically in the *nsrR* mutant compared to the WT strain (Table 16). Both genes encoding the vitamin B12 dependent (MetH) and the independent (MetE) were slightly up-regulated anaerobically in both strains; although less in $\Delta nsrR$. Thus, it seems that the higher NO levels, seen in the *nsrR* mutant, do not destroy the vitamin B12 pool or at least they interfere in a non-crucial manner. The two explanations for this are a very quick and sufficient NO detoxification machinery and most likely the fact that the growth media contains casamino acids, which contain methionine.

sRNA

The OmpX-repressor CyaR, which was shown to be up-regulated anaerobically in the WT strain (Table 14), was down-regulated under both aerobic as well as anaerobic conditions in the *nsrR* mutant (Table 15 and Table 16). Another

sRNA that was transcribed differently in the WT and the *nsrR* mutant strains was the pathogenicity island associated *isrA* with unknown function. Aerobically it was approximately 18-fold higher expressed in the *nsrR* mutant (Table 15), but unlike being up-regulated during anaerobiosis as in the WT strain it was down-regulated in $\Delta nsrR$ in the absence of O₂. Most of the sRNAs that were shown to be higher transcribed anaerobically in the *nsrR* mutant (Table 16) were slightly less down-regulated. The *glnL* gene was one of the exceptions that was higher up-regulated.

Other functional categories

Interesting differences between the two strains were also seen in the functional categories: functional unknowns, pathogenicity and cytochrome c biogenesis (Table 15 and Table 16). It was noticeable that many of the genes that were down-regulated in the *nsrR* mutant are associated with fimbriae; proteins important for cell adhesion. These included genes of the *fim* operon (*fimC*, *fimH*, *fimL* and *fimY*) and genes essential for the biogenesis of plasmid-encoded fimbriae (*pefB* and *srgA*) (Bouwman et al., 2003). This agrees with an *E. coli* study which showed that NO inhibits the expression of certain pathogenicity island-associated genes essential for cell adherence and that *nsrR* is a positive regulator of these operons (Branchu et al., 2014). Furthermore, all genes that were classified to be involved in cytochrome c biogenesis were up-regulated anaerobically in the *nsrR* mutant (Figure 36). The absence of NsrR resulted in higher anaerobic expression levels in some of these (*ccmA*, *ccmC* and *ccmE*) (Table 16). The excess amount of cytochrome c was presumably required by the cytochrome c nitrite reductase NrfA, which was shown to be more highly expressed.

5.5 Discussion

In this study a combined physiological (chemostat) and transcriptional approach (qRT-PCR and Microarray analysis) was used to characterize the contribution of NsrR regulon to endogenous N₂O production of anaerobically grown *Salmonella*

chemostat cultures. To our knowledge, this has been the first study to do so, since other transcriptional studies have been performed in different organisms, aerobically or they looked at the response of NsrR regulated genes to exogenic NO sources. First, continuous chemostat experiments were performed with the *Salmonella* SL1344 WT and $\Delta nsrR$ strain in order to compare their nitrate respiration products; nitrate, nitrite and N₂O under both nitrate-sufficient/glycerol-limited and nitrate-limited/glycerol-sufficient growth. The absence of NsrR resulted in higher N₂O levels under nitrate-sufficient conditions (Figure 29 and Figure 30). To our surprise, the difference was even more striking during glycerol-sufficient growth. In contrast to the WT strain, relatively high levels of both N₂O and nitrite accumulated during the transition phase in the *nsrR* mutant and stayed high throughout the experiment (Figure 32 and Figure 33). This behaviour resembled more that of a culture grown under nitrate-sufficiency and not nitrate-limitation. The same was true for the growth pattern of $\Delta nsrR$. Thus, in order to find an explanation for this, transcriptional analyses were performed. Initial tests, using qRT-PCR, showed that all genes known to be important for anaerobic nitrate respiration and NO detoxification were higher up-regulated anaerobically in the *nsrR* mutant (Figure 31 and Figure 34). This was confirmed by later microarray experiments which were performed under glycerol-sufficient growth conditions, as the differences between the two strains were more prominent. Furthermore, the microarrays revealed that there were significant differences in genes belonging to functional groups other than the nitrate metabolism (e.g. oxidative stress, pathogenicity, iron and sulphur metabolism, vitamin B12 metabolism or sRNAs) (Figures 35-38). However, most genes had the same transcriptional pattern (up-/down regulation +O₂ vs. -O₂ growth) compared to the wild type and only differed in their transcriptional level. Since the most transcriptional differences were shown to fall under the nitrate metabolism category and because one of the aims was to determine the reason for the higher N₂O levels of the *nsrR* mutant, the remaining discussion will now focus on the genes of the nitrate metabolism.

The *narGHJI* and *nirBCD* operons, which are not under the control of NsrR, were only found to be induced in the *nsrR* mutant (Table 14 and Appendix E). Furthermore, all NsrR regulated genes, including genes of the *nap* and the *nrf*

operon were higher expressed anaerobically in (Table 16). Interestingly, all the NsrR-regulated genes that were previously shown to be induced aerobically in *Salmonella* (Karlinsey et al., 2012), were the only ones that were higher expressed under both aerobic and anaerobically in the absence of *nsrR* (Table 17 and Figure 39). However, among these only *hmpA*, *hcp* and *hcr* were up-regulated under anaerobic conditions in $\Delta nsrR$, while *STM1808*, *yeaR*, *yoaG*, *ygbA* and *ytfE* were down-regulated. Although *hmpA* and *hcp-hcr* were both up-regulated, their expression pattern differed. The *hmpA* gene was completely deregulated by the derepression of NsrR, since it was constitutively expressed; presumably causing the slight growth defect (see section 5.4.3.2). In contrast, the *hcp-hcr* operon had relatively low aerobic expression levels (REL = 1.5-3) compared to that of *hmpA* (REL = 40), but it was shown to be maximally induced during anaerobic conditions (Table 17). This agrees with an *E. coli* study, which further showed that the presence of nitrite is required for its induction; conditions that were only found in the *nsrR* mutant (Figure 33) (Chismon et al., 2010). Activation by NarXL and NarQP further contribute to the high expression levels of *hcp-hcr*. Thus, the question arises of what is the role of this operon. It has been suggested to be involved in aerobic NO detoxification but sufficient proof was missing (Karlinsey et al., 2012, Cole, 2012) and therefore it would also be possible that Hcp-Hcr are involved in NO production. In addition, two of the down-regulated genes, *STM1808* and *ytfE*, also seem to be deregulated by NsrR as they had the highest aerobic RELs detected (108 and 62, respectively). Furthermore, they were both approximately 14-fold higher expressed anaerobically in the *nsrR* mutant compared to the WT strain (Table 17). Two hypotheses exist that could explain the higher N₂O levels of the *nsrR* mutant: 1. NarG produces NO, which in turn becomes detoxified to N₂O by HmpA and possibly Hcp-Hcr. 2. NO is produced by NarG and Hcp-Hcr, which is then converted to N₂O by HmpA. Either way, it is clear that these operons play an important role in anaerobic nitrate respiration. This was confirmed by nitrate-limited Hungate batch culture experiments, which showed that a single deletion of *hmpA*, *hcp* or *hcr* resulted in significantly lower levels of N₂O compared to the WT strain (Chapter 6; Figure 41). The anaerobic NO detoxification enzyme, NorVW, is most likely not involved in this process, as it was only poorly expressed during glycerol-sufficient growth conditions. Considering the fact that

the high N₂O levels are not just produced in the *nsrR* mutant but also stay level throughout the experiment (Figure 33), it is possible that a weak N₂OR also contributes to this. Chapter 4 showed that it is likely that *Salmonella* possesses a yet unknown N₂OR and that the encoding gene might belong to the NsrR regulon (lower N₂O reduction rate in $\Delta nsrR$ vs. WT; (Table 12). If this is the case, it must be a gene that is activated by NsrR, so it becomes inactivated in its absence and which is up-regulated anaerobically in the WT strain. However, this gene was not identified in this study. In conclusion, this study showed that, apart from NarG, genes of the NsrR regulon caused the differences seen in N₂O production. Thus, mutagenesis experiments were performed next, to determine the contribution of each of these genes.

5.6 Future work

For publication purposes, some of the experiments would need to be repeated, such as the nitrate-sufficient chemostat cultures and the microarray analysis. In addition, it would be interesting to see if a *Salmonella* culture with an overexpressed HmpA would result in higher N₂O levels compared to a wild type strain under glycerol-sufficient growth conditions. The same should be done for *hcp-hcr* and *STM1808*. Another way to test the contribution of each of these genes is mutagenesis experiments, which were performed in the next chapter. Furthermore, chemostat experiments with a $\Delta hcp-hcr$ single mutant or *hcp-hcr* double mutants ($\Delta narG\Delta hcp-hcr$, $\Delta napD\Delta hcp-hcr$ or $\Delta hmpA\Delta hcp-hcr$) could explain the role of Hcp-Hcr during anaerobic nitrate respiration by comparing their nitrogen metabolites (NO₃⁻ NO₂⁻ N₂O).

**6 The contribution of the NsrR regulon to
endogenous N₂O production in *Salmonella*
Typhimurium**

6.1 Introduction

Previous chapters have indicated a potential role for NsrR regulated genes in the possible reduction of N₂O in *Salmonella* Typhimurium. Transcriptional studies have shown that many of the NsrR regulon members, including *hmpA*, *hcp*, *hcr*, *ytfE*, *ygbA*, *yeaR*, *yoaG*, *tehB* and *STM1808*, are induced in response to exogenous NO (Bang et al., 2006, Filenko et al., 2007, Lin et al., 2007, Gilberthorpe et al., 2007, Bodenmiller and Spiro, 2006, Karlinsey et al., 2012, Cole, 2012). However, their role in anaerobic nitrate respiration and nitrosative stress response has been poorly characterised (see Chapters 1 and 5 for more information). Thus, in this chapter we performed a phenotypic characterisation to elucidate the contribution of individual NsrR regulated genes to endogenous N₂O production of *Salmonella* during anaerobic nitrate respiration. Hungate batch culture experiments were used to allow an efficient screening of single, double and triple mutants under both nitrate-sufficient/glycerol-limited and glycerol-sufficient/nitrate-limited growth conditions.

6.2 Aim

The aim of this study was to determine the contribution of *hmpA*, *hcp*, *hcr*, *ytfE*, *ygbA*, *yeaR*, *yoaG*, *tehB* and *STM1808* to endogenous N₂O production by *Salmonella* Typhimurium during anaerobic nitrate respiration.

6.3 Experimental design

6.3.1 Anaerobic Hungate batch of NsrR regulon members

S. Typhimurium SL1344 WT and mutants of the NsrR regulon were cultured anaerobically in Hungate tubes for 24 hours as described in section 2.7. All SL1344 WT background mutants were kindly provided by Dr Anke Arkenberg and Dr Corrine Appia-Ayme (Lab culture collection). All SL1344 Δ *nsrR*

background mutants were created by Tom Williams (undergraduate student/Rowley Lab) using phage transduction as described in section 2.15. The *ΔnarG Δhcp* and the *ΔnapDA Δhcp* double mutants were created in this study using phage transduction as described in section 2.15. The MGN media was either supplemented with 22 mM nitrate and 5 mM glycerol to achieve nitrate-sufficient/glycerol-limited growth conditions or with 20 mM glycerol and 5 mM nitrate to achieve nitrate-limited/glycerol-sufficient growth. The OD_{590nm} has been determined spectrophotometrically as described in section 2.9. N₂O was determined by GC analysis as described in section 2.11. Experiments were performed in triplicate.

6.4 Results

6.4.1 The contribution of the Salmonella NsrR regulon to N₂O production

S. Typhimurium SL1344 WT and mutant strains, belonging to the NsrR regulon, were cultured anaerobically in Hungate tubes for 24 hours. All tested strains followed a similar growth pattern and reached a final OD (at 24 h post inoculation) of 0.22 ± 0.01 when grown under N⁺/G⁻ conditions or 0.18 ± 0.01 under G⁺/N⁻ growth conditions (Appendix F). SL1344 *Δhcp* and *Δhcr* were an exception to this as they had a slightly lower final OD of 0.19 ± 0.01 , when grown under N⁺/G⁻ conditions. The N₂O production of different mutants of the NsrR regulon at 24 hours post inoculation was expressed as the percentage of the relative N₂O levels (μM per OD unit) compared to the WT strain (Figure 40 and Figure 41).

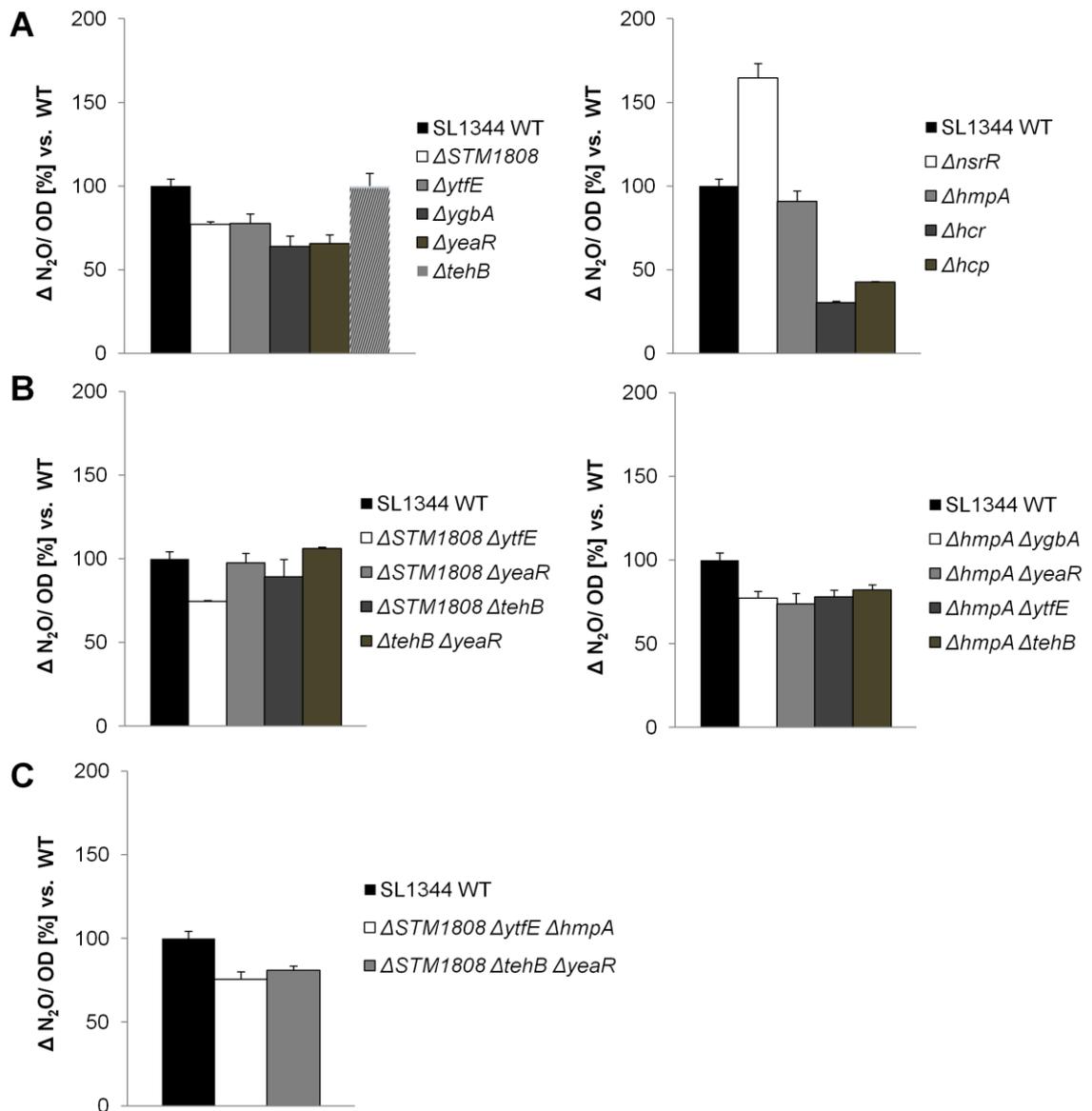


Figure 40 Nitrous oxide production of nitrate-sufficient anaerobic batch cultures of selected *Salmonella* NsrR regulon members.

Panel A: Nitrous oxide production (measured at 24 hours post inoculation) of single deletion mutants, expressed as the percentage of the relative N_2O levels [$\mu M/OD$ unit] compared to the WT strain. Panel B and C: N_2O production levels of double and triple mutants, respectively. The cultures were grown in Hungate tubes in 10 mL MGN (N^+/G^-) medium, inoculated with 2% of a MGN overnight. Data are mean \pm standard deviation ($n=3$).

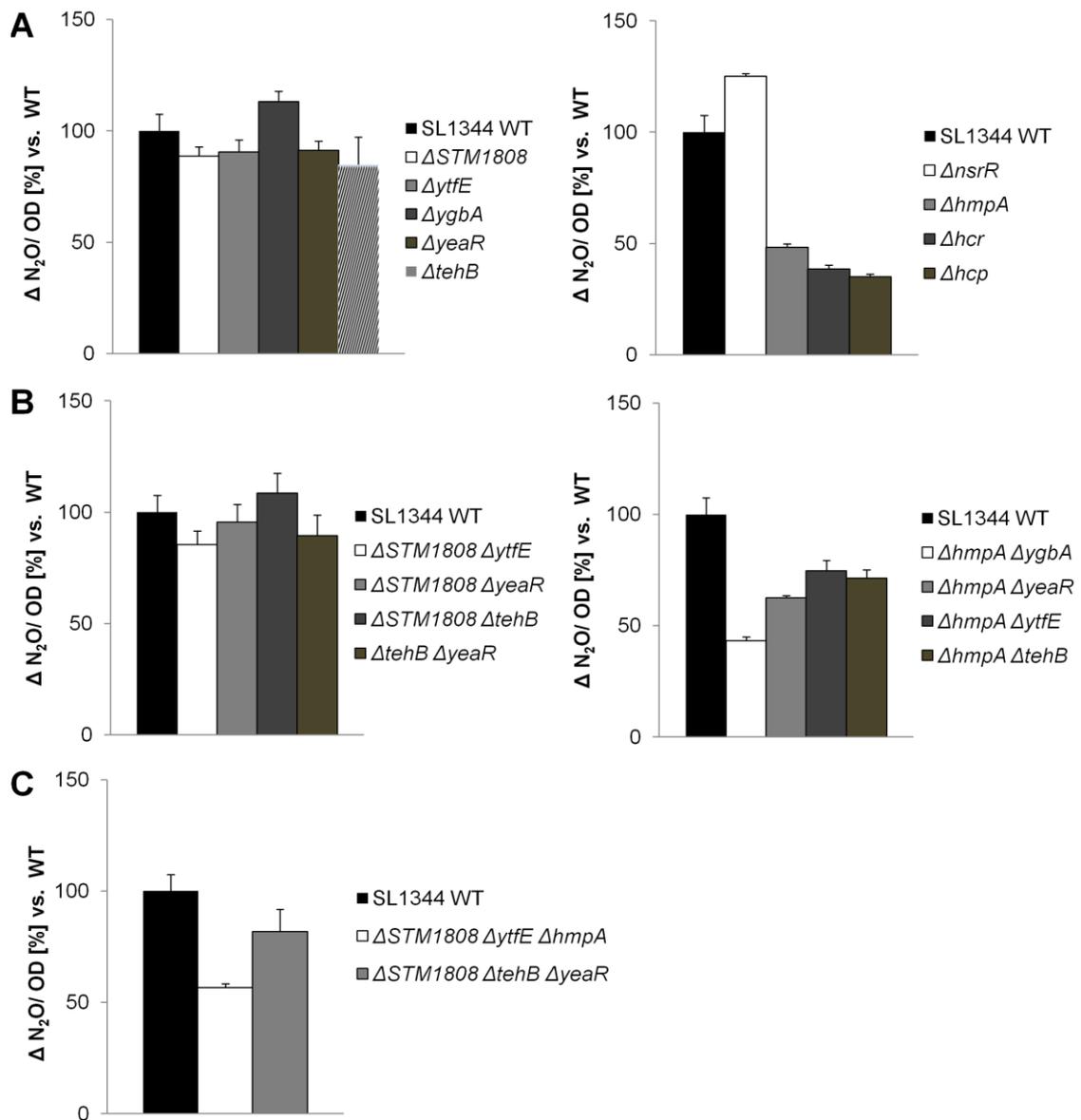


Figure 41 Nitrous oxide production of nitrate-limited anaerobic batch cultures of selected *Salmonella* NsrR regulon members.

Panel A: Nitrous oxide production (measured at 24 hours post inoculation) of single deletion mutants, expressed as the percentage of the relative N_2O levels [$\mu M/OD$ unit] compared to the WT strain. Panel B and C: N_2O production levels of double and triple mutants, respectively. The cultures were grown in Hungate tubes in 10 mL MGN (G^+/N^-) medium, inoculated with 2% of a MGN overnight. Data are mean \pm standard deviation ($n=3$).

The absolute N₂O levels of SL1344 WT are approximately 13 μM under nitrate-sufficient growth conditions (Chapter 3) and reached up to approximately 20 μM during glycerol sufficiency in this study. This was surprising since chemostat experiments showed that high N₂O levels require nitrate-sufficient/glycerol-limited growth conditions (Chapter 3). However, it must be considered that the Hungate batch culture environment inflicts more stress on the organism, such as a shorter aerobic phase or accumulation of toxic by-products, compared to the controlled chemostat environment. Therefore, it is possible that *Salmonella* uses the limited nitrate available more efficiently under G⁺/N⁻ growth conditions, which in turn results in higher N₂O levels. This might also explain the lower final OD of cells grown under G⁺/N⁻ conditions (Appendix F).

Figures 40 and Figure 41 provide an overview of the contribution of each tested NsrR regulon gene to the N₂O production of *Salmonella* under N⁺/G⁻ and G⁺/N⁻ growth conditions, respectively. Since all of these genes are known to be repressed by NsrR, *nsrR* background double and triple mutants have been tested afterwards to determine the impact of the gene deletions on the N₂O levels in the absence of NsrR (Figure 42). In agreement with the chemostat results (Figures 29-30 and Figures 32-33), Hungate batch culture experiments showed that the *nsrR* mutant achieved higher N₂O levels compared to that of the WT strain under both growth conditions. Furthermore, a single gene deletion in any of the tested NsrR regulon members, apart from *tehB*, resulted in decreased N₂O levels in at least one of the two growth conditions. HmpA seems to be important for NO detoxification in *Salmonella* during G⁺/N⁻ growth conditions (Figure 41), since the N₂O levels of $\Delta hmpA$ were approximately 50% lower compared to that of the WT strain, but it plays only a minor role during nitrate-sufficient growth (Figure 40), where there is no detectable difference. The reason for this is that HmpA is strongly repressed by its principal regulator NsrR. However, in the absence of NsrR, i.e. when *hmpA* is overexpressed (see Chapter 5), it contributes up to 25% of the N₂O levels produced by the *nsrR* mutant during nitrate-sufficient growth (Figure 42A) and approximately 50% during nitrate limitation (Figure 42B).

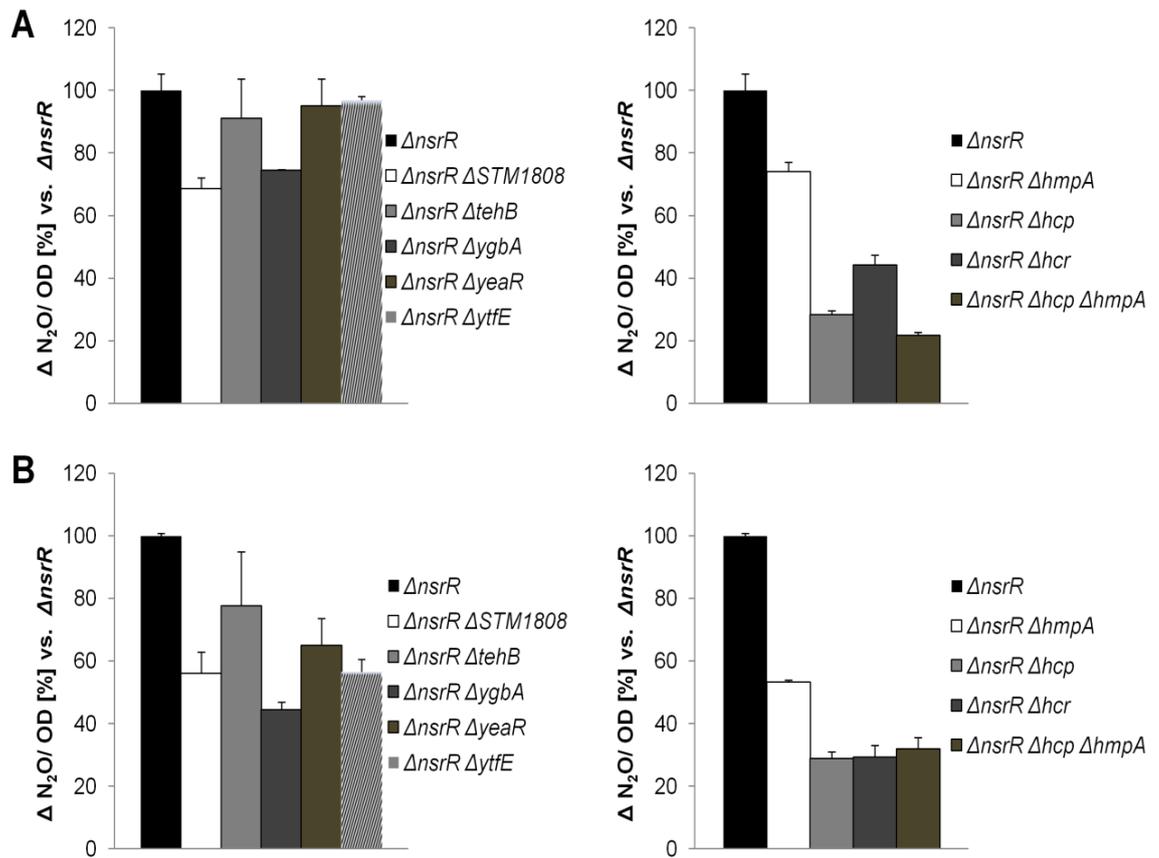


Figure 42 Nitrous oxide production of anaerobic batch cultures of selected *Salmonella AnsrR* mutants.

Panel A: Nitrous oxide production (measured at 24 hours post inoculation) of $\Delta nsrR$ background mutants grown under nitrate-sufficient/glycerol-limited conditions; expressed as the percentage of the relative N₂O levels [$\mu\text{M}/\text{OD}$ unit] compared to the *nsrR* mutant. Panel B: N₂O production levels of $\Delta nsrR$ background mutants grown under G⁺/N⁻ conditions. The cultures were grown in Hungate tubes in 10 mL MGN (A: N⁺/G⁻ and B: G⁺/N⁻) medium, inoculated with 2% of a MGN overnight. Data are mean \pm standard deviation (n=3).

Since the contribution of HmpA to *Salmonella*'s N₂O production was found to be very limited during nitrate sufficient growth (no difference compared to WT, Figure 40A), the loss of the other genes in the *hmpA* double mutants must have been the cause for the 20% lower N₂O levels (Figure 40B). In contrast, the N₂O levels of the *hmpA* double mutants grown under G⁺/N⁻ conditions were predominantly determined by the loss of *hmpA* (Figure 41B). STM1808 and YgbA had an impact on N₂O levels under N⁺/G⁻ conditions but, within errors, there was no detectable difference in comparison to the WT strain during glycerol-sufficient growth. This might be because of their strong regulation by NsrR as they were found to play an important role during anaerobic nitrate respiration under both growth conditions in the $\Delta nsrR$ background experiments (Figure 42). Since NsrR is inactive during nitrate-sufficient conditions, NsrR repressed genes become strongly derepressed. In addition, STM1808 and *ygbA* were only poorly expressed during G⁺/N⁻ growth conditions (Microarray results), but they became highly transcribed in the absence of NsrR (Table 17). Out of all STM1808 double mutants ($\Delta STM1808\Delta ytfE$, $\Delta STM1808\Delta yeaR$ and $\Delta STM1808\Delta tehB$) only $\Delta STM1808\Delta ytfE$ produced lower amounts of N₂O compared to the WT strain under N⁺/G⁻ conditions. None of these had an obvious contribution to N₂O production during G⁺/N⁻ growth conditions. The $\Delta ytfE$ and $\Delta yeaR$ strains behaved very similar to the $\Delta STM1808$ and $\Delta ygbA$ single mutants, with the difference that in the absence of NsrR they only have an influence on the N₂O levels under glycerol-sufficient conditions (Figure 42). As previously mentioned, TehB does not seem to be involved in the N₂O production of *Salmonella* at all, since even in the *nsrR* background mutant the deletion of *tehB* resulted in a maximal reduction of 10% N₂O under G⁺/N⁻ conditions (Figure 42). This agrees with its relatively low expression levels during anaerobiosis.

The two most interesting mutants were Δhcp and Δhcr , which had by far the lowest N₂O levels determined under both conditions, and irrespective of the presence of NsrR (Figures 40-42). Nonetheless in the *nsrR* background, their absence had the biggest impact on N₂O production in *Salmonella*. This agrees with the microarray data that showed that *hcp* and *hcr* were the highest induced genes (section 5.4.3 and Table 17). A deletion of three genes resulted in a

cumulative effect. Under N^+/G^- conditions, the $\Delta STM1808\Delta ytfE\Delta hmpA$ strain had a similar N_2O level compared to that of the $\Delta STM1808\Delta ytfE$, since the deletion of *hmpA* had almost no effect. In contrast, during G^+/N^- conditions where *hmpA* had a big impact on the N_2O levels, a reduction in N_2O similar to that seen in $\Delta hmpA$ was detectable.

6.4.2 N_2O production of nitrate-sufficient batch cultures of *Salmonella nar*, *nap* and *hcp* mutants

As shown in the previous section, Hcp and Hcr have an immense impact on the N_2O production of *Salmonella*. Although they have a suggested function in NO detoxification (Cole, 2012, Karlinsey et al., 2012), this hypothesis lacks sufficient evidence. It would also be possible that they are instead both involved in NO production. To investigate this further, $\Delta napDA\Delta hcp$ and $\Delta narG\Delta hcp$ double mutants have been tested (Figure 43). The $\Delta napDA$, Δhcp and the $\Delta napDA\Delta hcp$ strains had a similar but slightly lower growth rate compared to the WT strain (Figure 43A). In contrast, $\Delta narG$ and $\Delta narG\Delta hcp$ had a severe growth defect. As known from our previous *Salmonella* study (Rowley et al., 2012) and Chapter 3, the nitrate reductase NarG is one of the key players for N_2O production during N^+/G^- growth conditions, while the loss of the periplasmic Nap has only a minor effect. The same is seen in this study for $\Delta narG$ but not $\Delta napDA$. The $\Delta narG$ strain had N_2O levels that were 50% lower (relative $[N_2O/OD]$; 70% absolute) compared to that of the WT strain. Although the effect of $\Delta napDA$ was weaker compared to that of $\Delta narG$, its deletion still resulted in approximately 25% lower N_2O levels compared to the WT strain (Figure 43B). The difference between the two studies is most likely a result of using batch vs. continuous cultures. The *hcp* mutant strain had similar low N_2O levels than $\Delta narG$, suggesting that it must be equally important for N_2O production. Furthermore it was very interesting to see that the N_2O levels of $\Delta napDA\Delta hcp$ were the same as that from the Δhcp single mutant. It was even more astonishing that the $\Delta narG\Delta hcp$ double mutant produced less than half of the amount of N_2O compared to $\Delta narG$. In fact, when looking at the absolute N_2O levels, it was obvious that $\Delta narG\Delta hcp$ barely produces any N_2O (1.3 μM compared to 14 μM in the WT) at all.

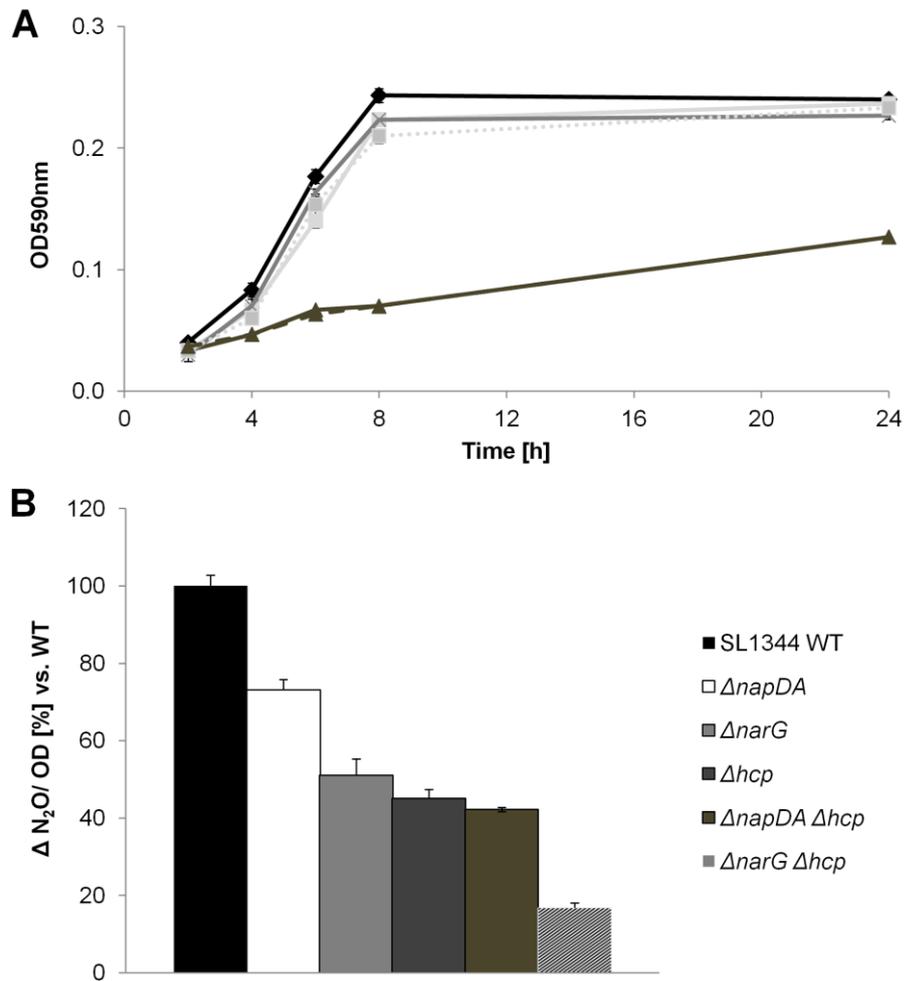


Figure 43 Nitrous oxide production of nitrate-sufficient anaerobic batch cultures of *Salmonella nar*, *nap* and *hcp* mutants.

Panel A: Anaerobic growth of *S. Typhimurium* SL1344 WT (black diamonds) $\Delta napDA$ (light grey squares), $\Delta narG$ (brown triangles), Δhcp (dark grey cross), $\Delta napDA \Delta hcp$ (light grey squares; dotted line) and $\Delta narG \Delta hcp$ (brown triangles; dashed line) in Hungate tubes. OD_{590nm} was taken at 2, 4, 6, 8 and 24 hours post inoculation. The cultures were grown in 10 mL MGN (N^+/G^-) medium, inoculated with 2% of a MGN overnight. Panel B: Nitrous oxide production (measured at 24 hours post inoculation) expressed as the percentage of the relative N_2O levels [$\mu M/OD$ unit] compared to the WT strain. Data are mean \pm standard error (n=3).

6.5 Discussion

In this study, mutagenesis experiments were used to determine the contribution of established (*hmpA* and *ytfE*) and less characterised NsrR regulon members (*hcp*, *hcr*, *STM1808*, *yeaR*, *ygbA* and *tehB*) to endogenous N₂O production in *Salmonella*. These were identified to be important for the high N₂O levels seen in an *nsrR* mutant, grown under glycerol-sufficient/nitrate-limited conditions (Chapter 5) and have been previously shown to respond to NO exposure. This study revealed that apart from *tehB*, each gene contributed to N₂O production in *Salmonella*, as gene deletions resulted in reduced N₂O levels in at least one of the two growth conditions (N⁺/G⁻ and G⁺/N⁻) (Figure 40 and Figure 41). The genes *STM1808*, *ytfE*, *ygbA* and *yeaR* were found to have an effect on the N₂O production only during N⁺/G⁻ growth conditions (Figure 40), while *hmpA* was predominately required when nitrate was limited (Figure 41). This was also reflected in the microarray data (Chapter 5), as *STM1808*, *ytfE*, *ygbA* and *yeaR* were poorly transcribed during G⁺/N⁻ growth conditions in the WT strain. The 50% reduction in N₂O production of $\Delta hmpA$ during G⁺/N⁻ revealed that HmpA is crucial for NO detoxification in *Salmonella* under these conditions, which supports earlier findings (Crawford and Goldberg, 1998, Gilberthorpe et al., 2007). The deletion of either *hcp* or *hcr* had the biggest impact on N₂O levels (up to 65% less compared to the WT strain), suggesting that they play a crucial role during anaerobic nitrate respiration in *Salmonella* (Figure 40 and Figure 41). The N₂O levels of *hmpA* double mutant were predominantly determined by one dominant gene; i.e. under N⁺/G⁻ conditions, where the deletion of *hmpA* had only a minor effect, N₂O levels were determined by the second gene deletion but during G⁺/N⁻ growth *hmpA* was the influencing gene (Figure 40B and Figure 41B). In contrast, in most of the other double and triple mutants the N₂O levels were a result of a cumulative effect of each gene deletion (Figure 40C and Figure 41C).

Since all tested genes are repressed by NsrR, *nsrR* background mutants were designed (Figure 42). This part of the study showed that *STM1808*, *ytfE*, *ygbA* and *yeaR* presumably require high levels of NO in order to become actively

involved in N₂O production/NO detoxification, because their deletion had a bigger impact during G⁺/N⁻ growth conditions, where higher N₂O levels were measured in Hungate batch culture experiments. This would make sense, because YtfE is known to be involved in the repair of NO damaged [Fe-S] clusters and should therefore be active when NO levels are high. The deletion of *hmpA*, *hcp* or *hcr* had again the biggest impact on N₂O levels. By creating $\Delta napDA\Delta hcp$ and $\Delta narG\Delta hcp$ double mutants, it was confirmed that the hybrid cluster protein plays a crucial role during anaerobic nitrate respiration in *Salmonella* (Figure 43). The $\Delta napDA\Delta hcp$ strain had 60% lower N₂O levels compared to the WT strain, which were similar to the values seen in a single *hcp* mutant. The deletion of both *narG* and *hcp* together resulted in almost no N₂O production at all (absolute N₂O levels: $\Delta narG\Delta hcp$ =1.3 μ M compared to 14 μ M in the WT). Although this study revealed the importance of the *hcp-hcr* operon for anaerobic nitrate respiration in *Salmonella*, their precise function (NO detoxification or NO production) still remains to be elucidated. Considering the fact that Δhcp and $\Delta napDA\Delta hcp$ had similar growth patterns to the WT strain, while $\Delta narG$ and $\Delta narG\Delta hcp$ had a severe growth defect, it seems more likely that *hcp* is involved in NO detoxification. This would agree with the suggestions of other studies (Cole, 2012, Karlinsey et al., 2012).

6.6 Future work

Most of the tested NsrR regulon genes have only been poorly characterised and their precise function in anaerobic nitrate respiration and NO detoxification still needs to be elucidated. Chemostat mutant experiments, coupled with protein film voltammetry would help to better understand the genes' functions. Hcp and Hcr are the most interesting candidates to start with, as they had the biggest contribution to the N₂O levels in *Salmonella*. With the help of chemostat experiments the metabolites nitrate, nitrite and N₂O could be determined, while protein film voltammetry would reveal information about the kinetics of the protein and its catalytic activity. Furthermore, with the help of a NO electrode (e.g. Modified Clark electrode as describes by Field *et al.* (2008) it could be

tested if *hcp* possesses a NO reductase activity. The reduction rate of the WT strain would be used as a positive control and medium or dead cells as negative controls. Assuming Δhcp possesses a NO reductase activity, this should be reflected in a slower NO reduction rate. NorVW could be used as an additional control. Furthermore, it would be interesting to see whether a $\Delta narG\Delta hcp\Delta norVW$ triple mutant would produce any N_2O under N^+/G^- growth conditions, if it would be able to grow at all. The same could be tested with a $\Delta napDA\Delta hcp\Delta hmpA$ mutant for G^+/N^- growth. Moreover, HPLC analysis could be used to test the nitrate consumption of *Salmonella* of Hungate batch cultures for both growth conditions. Thereby, it would be possible to explain why G^+/N^- growth conditions resulted in higher N_2O levels compared to N^+/G^- growth conditions in Hungate batch culture but not in chemostat experiments.

7 General discussion

Since each chapter has been discussed individually, this general discussion is used to highlight the major outcomes of this study and to put these into context with their impact on *Salmonella* research.

7.1 Context

Salmonella spp. are harmful bacterial pathogens that cause a spectrum of diseases in humans and animals, ranging from gastroenteritis (food poisoning) to enteric (Typhoid) fever. Among other limitations, current typhoid fever vaccines do not provide a life-long protection against *Salmonella* Typhi. Furthermore, the misuse of antibiotics has resulted in the evolution of multidrug resistant *Salmonella* strains that can turn a usually self-limiting gastroenteritis into a life-threatening disease in the immunocompromised host, such as the elderly and infants. Thus, there is an urgent need for new treatments and a better understanding of *Salmonella*'s virulence factors and pathogenic mechanisms.

Salmonella encounters various stresses during its life cycle in the host (human and animals) and non-host (soil, water and industrial) environments (Runkel et al., 2013). Nitrosative stress in form of RNS, such as the potent cytotoxin NO, presents one of these stresses. *Salmonella* is exposed to exogenous NO, produced by activated macrophages as part of the host immune response as well as endogenous NO, produced during anaerobic nitrate respiration. Thus, sophisticated defence mechanisms are required to survive the detrimental effects of NO. *Salmonella* and *E. coli* employ three enzymes (HmpA, NrfA and NorVW) that detoxify NO to less toxic compounds, including the neuropharmacological agent and greenhouse gas N₂O. The production of NO and N₂O has predominantly been studied in denitrifying soil bacteria, but it has been widely neglected in enteric bacteria. A better understanding of the mechanisms involved could provide new insights into host-pathogen interactions, which might lead to new treatment strategies for *Salmonella* infections, help to increase food safety and potentially help to reduce global warming.

Thus, the overall aim of this thesis was to enhance the understanding of nitrate respiration processes in enteric bacteria by comparing the physiological and molecular mechanisms involved in endogenous NO production and detoxification in the pathogenic *Salmonella enterica* serovar Typhimurium and laboratory *Escherichia Coli* strains.

7.2 *Salmonella* and the host microbiota

Enteric bacteria benefit from the ability to reduce nitrogen species for energy purposes and protection against RNS. NarG was found to be the major enzyme important for anaerobic nitrate respiration during a high nitrate to carbon ratio, resulting in high N₂O levels in *Salmonella* (Rowley et al., 2012). In this study we compared the nitrate respiration metabolites of two closely related organisms *Salmonella* and *E. coli*, which possess the same known nitrate respiration and NO detoxification systems and revealed that there are significant differences between the two genera and even between different *E. coli* strains, in relation to N₂O production. The reason for these differences was found to be at the transcriptional level, with *narG* expression levels having the biggest impact (Chapter 3). Consequently the question arises of whether there is a general correlation between pathogenicity and high N₂O production. This question has not been addressed here, but the basis for investigating this principle has been set. Answering this question would be very helpful in expanding our understanding of the importance of metabolic flux in host-pathogen interactions and would urge the development of new antimicrobial treatment strategies. *Haemophilus influenzae*, a small non-motile Gram-negative pathogenic bacterium, would be the ideal follow up research organism as its whole genome has been sequenced and because it lacks the Nar system.

NarG is responsible for the conversion of nitrate via nitrite down to NO (Rowley et al., 2012), but it requires the help of HmpA and NorVW to complete the process and detoxify NO to N₂O. Recent studies showed that established

(*hmpA*, *ytfE*) as well as less characterised (*hcp*, *hcr*, *STM1808*, *yeaR*, *yoaG*, *ygbA* and *tehB*) genes of the NsrR regulon have an important function in anaerobic nitrate respiration and in the defence against nitrosative stress (Bang et al., 2006, Filenko et al., 2007, Lin et al., 2007, Gilberthorpe et al., 2007, Bodenmiller and Spiro, 2006, Karlinsey et al., 2012, Cole, 2012). In this study, we determined for the first time the contribution of each of these genes to endogenous N₂O production in *Salmonella* and revealed that HmpA and the Hcp-Hcr operon are both crucial for high N₂O levels (Chapter 5 and 6). While HmpA is known to be the primary enzyme for the detoxification of NO to nitrate aerobically, its importance during anaerobic growth is still in debate (Gardner and Gardner, 2002, Hutchings et al., 2002, Poock, 2002, Mills et al., 2008, Crawford and Goldberg, 1998, Gilberthorpe et al., 2007). However, this study clearly showed that the loss of *hmpA* resulted in a 50% decrease in N₂O levels under glycerol-sufficient/nitrate limited conditions and thus agrees with earlier findings that HmpA can play an important role in anaerobic NO detoxification (Crawford and Goldberg, 1998, Gilberthorpe et al., 2007). In contrast, Hcp-Hcr was shown to be important during nitrate-sufficient/glycerol-limited as well as glycerol-sufficient/nitrate limited growth conditions (Chapter 6). Its precise role still needs to be elucidated but based on the results of this and other studies (Cole, 2012, Karlinsey et al., 2012), Hcp and Hcr seem to be involved in aerobic as well as anaerobic NO detoxification.

Consequently, *Salmonella* possesses several mechanisms to produce high levels of N₂O. Thus, based on the first question of the correlation of high N₂O production and pathogenicity, a subsequent question needs to be asked: What role does the high N₂O production of *Salmonella* play in the human gut and more specifically, are there any effects on the commensal flora or indeed the host? It is known that increased gastric NO, derived from either the acidification of dietary nitrate in the stomach or from the host's immune response during an infection, provides a defence mechanism against pathogenic bacteria. Nonetheless, high levels of NO were also found to promote the growth of facultative anaerobic Enterobacteriaceae, while depleting the amount of fermenting gut microbes (Stecher et al., 2007, Winter et al., 2010, Winter et al., 2013). In the gut, host-derived NO can be converted to nitrate via interactions

with ROS, which are produced during inflammation, and subsequently be used by *Salmonella* and *E. coli* for anaerobic nitrate respiration (Winter et al., 2013, Runkel et al., 2013). *Salmonella* uses the ability to respire nitrate and to detoxify NO to its advantage and actively triggers gut inflammation by the use of the T3SS effector SopE to boost its own growth and concurrently outcompete the intestinal microbiota (Lopez et al., 2012, Stecher et al., 2007, Winter et al., 2010). Lopez et al. (2012) further showed that mutations in the nitrate respiration genes; *narG*, *narZ* and *napA* resulted in a loss of the advantage gained by SopE (Lopez et al., 2012, Runkel et al., 2013). In addition, *Salmonella* has another growth advantage in the gut; its ability to use tetrathionate as an alternative electron acceptor for energy generation (Winter et al., 2010). Nonetheless, nitrate is the preferred electron acceptor during anaerobiosis and suppresses the genes required for tetrathionate respiration (Lopez et al., 2012, Runkel et al., 2013).

From the discussion above it is clear that anaerobic nitrate respiration provides *Salmonella* with a growth advantage over the commensal gut flora. However, there is another conceivable strategy that *Salmonella* could use to win the battle. Under nitrate-sufficient conditions *Salmonella* produces a high amount of N₂O (mM range). *Paracoccus* studies have shown that N₂O exhibits a cytotoxic effect at extracellular concentrations as low as 0.1 mmol/L by binding to and inactivating the vitamin B12 pool, which is essential for many proteins and required for methionine and DNA synthesis (Sullivan et al., 2013). Similar observations have been made in *E. coli* (Drummond and Matthews, 1994). Since *Salmonella* possesses two methionine synthases, a vitamin B12-dependent (MetH) as well as a vitamin B12-independent (MetE), it could outcompete all gut bacteria that lack the vitamin B12-independent pathway. This could potentially also lead to a vitamin B12 deficiency in humans that are unable to synthesize vitamin B12 themselves and therefore require the uptake of vitamin B12 via their diet. This theory seems plausible since vitamin B12 deficiency has been previously reported as a consequence of N₂O-based anaesthetics, but further research will be needed for validation (Sullivan et al., 2013, Badner et al., 2000).

7.3 *Salmonella* and the non-host environment

The ability to respire nitrate is also advantageous for *Salmonella* outside the host and facilitates survival in diverse soil, water and industrial environments. The Centre for Disease Control and Prevention (CDC) has estimated that 95% of *Salmonella* infections originate from food borne sources and that there is a continuous rise in numbers of fresh produce-based outbreaks (Fatica and Schneider, 2011). Common vehicles for transmission are contaminated eggs and meat products (especially poultry and pork) but even fruits and vegetables, in particular leafy greens and tomatoes can be affected, since *Salmonella* has a high prevalence in the farm environment e.g. as a common inhabitant of swine intestines (Gu et al., 2011, Fatica and Schneider, 2011, Mühlig et al., 2014, Islam et al., 2004, Baer et al., 2013). These contaminations often originate from irrigation water and contaminated manure that is spread on the fields or from fecal matter and direct contact of roaming animals including birds, rodents, reptiles and amphibians (Fatica and Schneider, 2011). *Salmonella* was found to be able to persist in soil treated with contaminated manure composts or irrigated water for more than 200 days (Islam et al., 2004).

In 2014, Mühlig *et al.* (2014) analyzed the nitrosative stress protection of *Salmonella* by HmpA, NorV and NrfA in raw sausages (Mühlig et al., 2014). This type of cured meat contains sodium nitrite as an antioxidant to extend the shelf-life and for antimicrobial purposes. *In vitro* experiments with 150 mg/L acidified nitrite demonstrated that HmpA provides protection against this stress and were supported by the transcriptional analysis that showed a strong up-regulation of *hmpA* but not *norV* or *nrfA* (Mühlig et al., 2014). However, *in situ* experiments failed to reveal a higher sensitivity of any of the mutants compared to the wild-type. Thus, it was speculated whether these systems act cooperatively in this environment or if there is a yet unknown mechanism involved (Mühlig et al., 2014). It would be interesting to see if the Hcp-Hcr complex could be the missing mechanism (see Chapters 5 and 6).

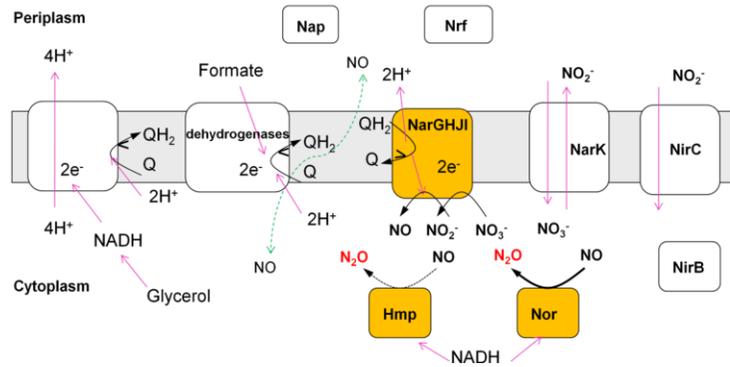
Furthermore, high nitrate levels in soil environments, which are predominately the cause of the excessive use of nitrate-containing fertilizers, pose a potential harm for the environment. A big concern is the production of the potent greenhouse gas N_2O by denitrifying soil microbes like *Paracoccus denitrificans*, which has a radiative potential that is 300 times higher than CO_2 (molecule per molecule) and an atmospheric lifetime of approximately 150 years (Richardson et al., 2009, Ravishankara et al., 2009). The Intergovernmental Panel on Climate Change (IPCC) estimated a 20% increase of atmospheric N_2O over the past century with an annual increase at a rate of 0.2-0.3% (Thomson et al., 2012). Polar ice sheets are huge inventories of nitrogen compounds (approximately 260 Tg N) and thus have a huge potential to further increase the atmospheric loading of N_2O (Wolff, 2013). Thus, N_2O is an important target for mitigation strategies to enhance the recovery of the ozone layer (Richardson et al., 2009, Ravishankara et al., 2009). This research focuses mainly on denitrifying soil bacteria but the impact of enteric bacteria to atmospheric N_2O is widely ignored. This seems bizarre, since denitrifying soil bacteria like *Paracoccus* possess the N_2O reductase NosZ. As a consequence, these bacteria would only produce high amounts of N_2O if the function of NosZ is inactivated, for instance in strongly acidic soil or by copper-limited conditions (Sullivan et al., 2013, Felgate et al., 2012). In contrast, enteric bacteria do not possess NosZ. In addition, this study demonstrated that *Salmonella* possesses only a weak N_2OR activity (Chapter 4) and that it is able to produce N_2O in the mM range under nitrate-sufficient conditions (Chapter 3); conditions that are found in agricultural soil that is enriched with nitrate-containing fertilizer and contaminated manure. Furthermore, similar to *Paracoccus*, the N_2O levels of *Salmonella* increased drastically at a pH of 5-6 (Chapter 4). Another environment where *Salmonella* can contribute to N_2O emissions is the wastewater treatment plant. N_2O emissions from wastewater treatment plants vary substantially and depend on efficient nitrogen removal (Law et al., 2012). Taken together, enteric bacteria like *Salmonella* should be included in future N_2O mitigation strategies and care should be taken to avoid excess use of nitrate-containing fertilizers on agricultural soil.

7.4 Concluding remarks

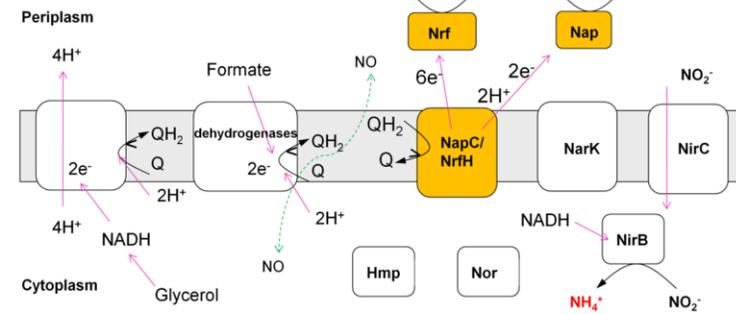
The work presented in this study expanded the understanding of the NO production and detoxification processes of *Salmonella* and *E. coli*. In particular, it emphasized the importance of NarG for anaerobic nitrate respiration and N₂O production, revealed the existence of a weak N₂OR in *Salmonella* and showed that additional enzymes, such as Hcp, have a significant contribution to the endogenous production of nitrous oxide. A summary of the anaerobic respiration pathway of *Salmonella*, which includes the results of this study, is given in Figure 44. Many possibilities exist for the use of this work for future research. Individual experiments are described in the future work section of each chapter and comprise three main areas: 1. Host-pathogen interactions: answering the questions of whether high N₂O production is correlated to pathogenicity and what effect does high N₂O levels have on microbial communities like the gut microbiota. 2. Identifying the N₂OR enzyme in *Salmonella*. 3. Establishing the role of Hcp and other poorly characterised enzymes that were shown to play an important role during anaerobic nitrate respiration and NO detoxification. This knowledge would have great applications in new treatment strategies for *Salmonella* infections, improved methods for food safety and greenhouse gas mitigation strategies. Although this research has contributed to archive these goals, it is still a long way to go to completely unravel all molecular and physiological functions of such globally important pathogens like *Salmonella*.

1. Established pathways

A. Nitrate sufficiency and glycerol limitation

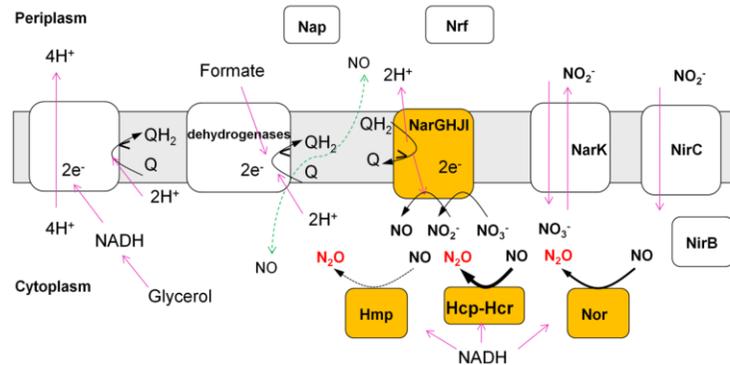


B. Nitrate limitation and glycerol sufficiency



2. New predicted pathways

A. Nitrate sufficiency and glycerol limitation



B. Nitrate limitation and glycerol sufficiency

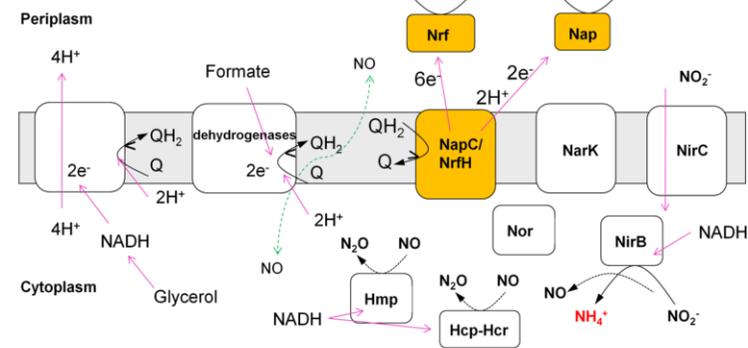


Figure 44 Modified from Rowley et al. (2012). Schemes for anaerobic nitrate respiration in *Salmonella Typhimurium* under different growth conditions. Panel 1 shows the well established nitrate respiration pathways while panel 2 highlights the importance of additional enzymes for these processes, as shown in this study. Growth conditions: A. nitrate-sufficiency/glycerol-limitation. B. nitrate-limitation/glycerol-sufficiency. Enzymes highlighted in yellow are the main reaction drivers under the given condition. Molecules highlighted in red are the predominant reaction products. Arrow thickness indicates the enzymes activity. The green dashed arrow shows that NO is able to freely diffuse across the membrane. New findings: 1. Hcp-Hcr is very important for N_2O production under both N^+/G^- and N^-/G^+ growth conditions, however its precise role still needs to be elucidated. 2. Hmp is important for N_2O production under N^-/G^+ growth conditions. The contribution of Hmp and Hcp-Hcr to N_2O production is even more prominent in the absence of the NsrR repressor.

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9 Appendix

A) Media composition and supplements

Luria Bertani (LB) Miller Broth (per L) (Bertani, 1951)

10.0 g Tryptone
5.0 g Yeast extract
10.0 g NaCl
Made up to 1 L with dH₂O and then autoclaved.

Luria Bertani (LB) Agar (per L)

10.0 g Tryptone
5.0 g Yeast extract
10.0 g NaCl
1.5 % Agar
Prepared LB broth. Added 3g of agar to a 500mL flask containing 200mL of LB broth and then autoclaved.

Minimal Media (MGN) (per L) modified from (Pope and Cole, 1982)

33.0 mM Dipotassium phosphate
30.0 mM Potassium dihydrogen phosphate
8.0 mM Ammonium sulphate
2.0 mM Tri-sodium citrate
0.20 mM Magnesium sulphate heptahydrate
0.160 mM Iron (II) chloride tetrahydrate
Made up to 1 L with dH₂O and added additions.

Additions before autoclavation:

0.001 mM Ammonium heptamolybdate (autoclaved)
0.001 mM Sodium selenate (autoclaved)
0.400 mM Magnesium chloride (autoclaved)
0.050 mM Manganese chloride tetrahydrate (autoclaved)
0.009 mM Calcium chloride dihydrate (autoclaved)

Supplements for (added before use only):

Nitrate sufficiency

22 mM Sodium nitrate (autoclaved)
5 mM Glycerol (autoclaved)
0.1 mg Casamino acids (filter sterilised)

Nitrate Limitation

5 mM Sodium nitrate (autoclaved)
20 mM Glycerol (autoclaved)
0.1 mg Casamino acids (filter sterilised)

***Paracoccus* Minimal Media (per L) modified from (Vishniac and Santer, 1957)**

29.0 mM Disodium hydrogen phosphate
11.0 mM Potassium dihydrogen phosphate
10.0 mM Ammonium chloride
0.4 mM Magnesium sulphate
Made up to 1 L with dH₂O and then autoclaved.

Supplements (added before use only):

20.0 mM Sodium nitrate (autoclaved)
5.0 mM Succinate (autoclaved)
2.0 mL Trace elements (autoclaved)

Trace element solution:

0.342 mM EDTA
0.015 mM Zinc sulfate heptahydrate
0.051 mM Manganese chloride tetrahydrate
0.036 mM Iron (II) sulfate heptahydrate
0.002 mM Ammonium heptamolybdate tetrahydrate
0.017 mM Copper (II) sulfate pentahydrate
0.014 mM Cobalt (II) chloride hexahydrate
0.100 mM Calcium chloride dihydrate

Green Plates (per L) (Maloy et al., 1996)

8.0 g Tryptone
1.0 g Yeast extract
5.0 g NaCl
1.5 % Agar

Prepared broth in 950 mL dH₂O. Added 3g of agar to a 500mL flask containing 190mL of LB broth and then autoclaved. Cooled down to 55°C and added additions.

Additions after autoclavation:

21 mL (4mL per flask) 40% glucose (autoclaved)
25 mL (5mL per flask) 2.5% alizarin yellow G (autoclaved; added hot)
3.3 mL (0.65mL per flask) 2% aniline blue (filter sterilised)

Green Indicator plates, used after P22 transduction to differentiate unstable P22 pseudo-lysogens from true lysogens were prepared as previously described (Maloy *et al.*, 1996).

Antibiotic Concentrations

100 $\mu\text{g mL}^{-1}$ Ampicillin
50 $\mu\text{g mL}^{-1}$ Kanamycin
10 $\mu\text{g mL}^{-1}$ Chloramphenicol

B) Hybridisation for microarrays

Solutions:

Wash 1 (1 litre)

20x SSPE	300 mL
20% N-Lauroylsarcosine	250 μL
Water	700 mL

Wash 2 (1 litre)

20x SSPE	3 mL
PEG200	1.8 mL
Water	995 mL

Reagents:

12X MES stock (100 ml)

MES free acid monohydrate	7.04 g
MES Sodium Salt	19.3 g

bring up to 100 ml Sigma water , 0.2 μm filter sterilize and store at 4 °C ;
1.22 M MES pH should be 6.5-6.7 (without adjustment)

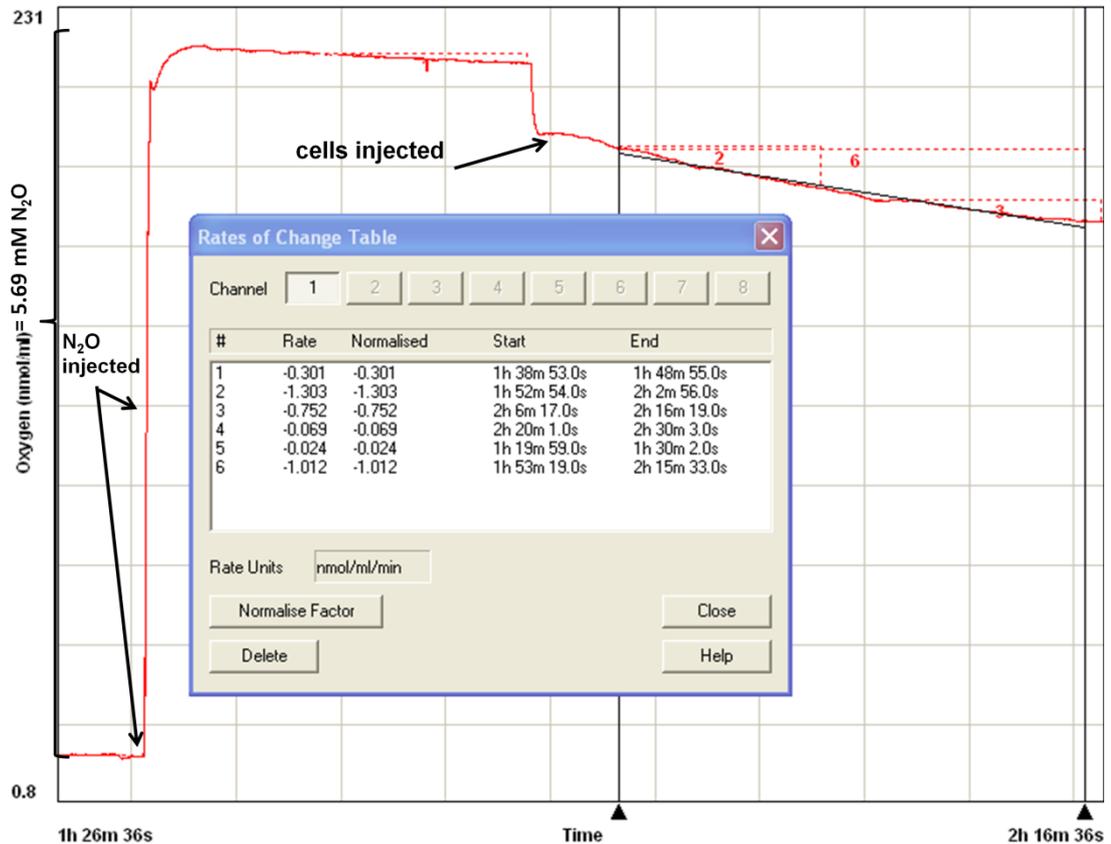
20X SSPE Buffer Recipe

Component	Amount	Stock 20X	Final 1X
NaCl	175.3 g	3 M	150 mM
NaH ₂ PO ₄ ·xH ₂ O	27.6 g	200 mM	10 mM
Na ₂ EDTA	7.4 g	200 mM	10 mM

bring up to 800 mL with H₂O, add NaOH to pH 7.4 (~27 mL/L of 10 M NaOH), autoclave for 20 min

C) N₂O electrode data (representative graphs)

SL1344 WT @ 37°C



For experimental procedure see Figure 28

Note:

Remember that the Clark electrode has been modified to detect N₂O in addition to O₂. However, the output signal of the Clark electrode is given in the unit “nmol O₂”, as this is the only possible output unit with the current software programme. Consequently, the output signal must be converted so that the apparent amount of O₂ can be converted to the amount of N₂O in solution.

Example calculation for SL1344 WT @ 37°C:

First the N₂O concentration present in the electrode chamber must be determined

Given:

Concentration saturated N₂O solution: 19.33 mM (determined via GC) = **C1**

Volume saturated N₂O solution injected: 2 x 0.3 mL = 0.6 mL = **V1**

Electrode chamber volume: 2.04 mL (2mL NO₃⁻-free media + 0.04 mL O₂ scrubbing system solutions) = **V2**

C2 = ? ; Formula : C1 * V1 = C2 *V2 → C2 = (C1 * V1) / *V2 = **5.69 mM N₂O**
Next the output signal (electrode) must be converted to N₂O to create a conversion factor.

Given:

Output signal (see graph): 219.59 \triangleq 5.69 mM N₂O → 219.59/ 5.69 = **38.59**
conversion factor

Mean of measured rate 2,3,6 (graph): 1.022 [nmol O₂ min⁻¹]

Background rate 1 (graph): 0.301 [nmol O₂ min⁻¹]

Formula:

N₂O reduction rate = Mean of measured rate 2,3,6 (graph) - Background rate 1 (graph) = **0.721 (from output signal)** [nmol O₂ min⁻¹]

Use conversion factor to convert the output signal [nmol O₂ min⁻¹] to N₂O levels [mM * min⁻¹].

0.721/38.59 = **0.0187 [mM * min⁻¹] N₂O**

Now the biomass has to be considered in order to determine the N₂O reduction rate per mg cells.

Given:

Drymass constant SL1344: 0.5021 (determined as described in section 2.13 [mg/mL])

OD_{590nm} SL1344: 0.150

Culture volume: 450 mL

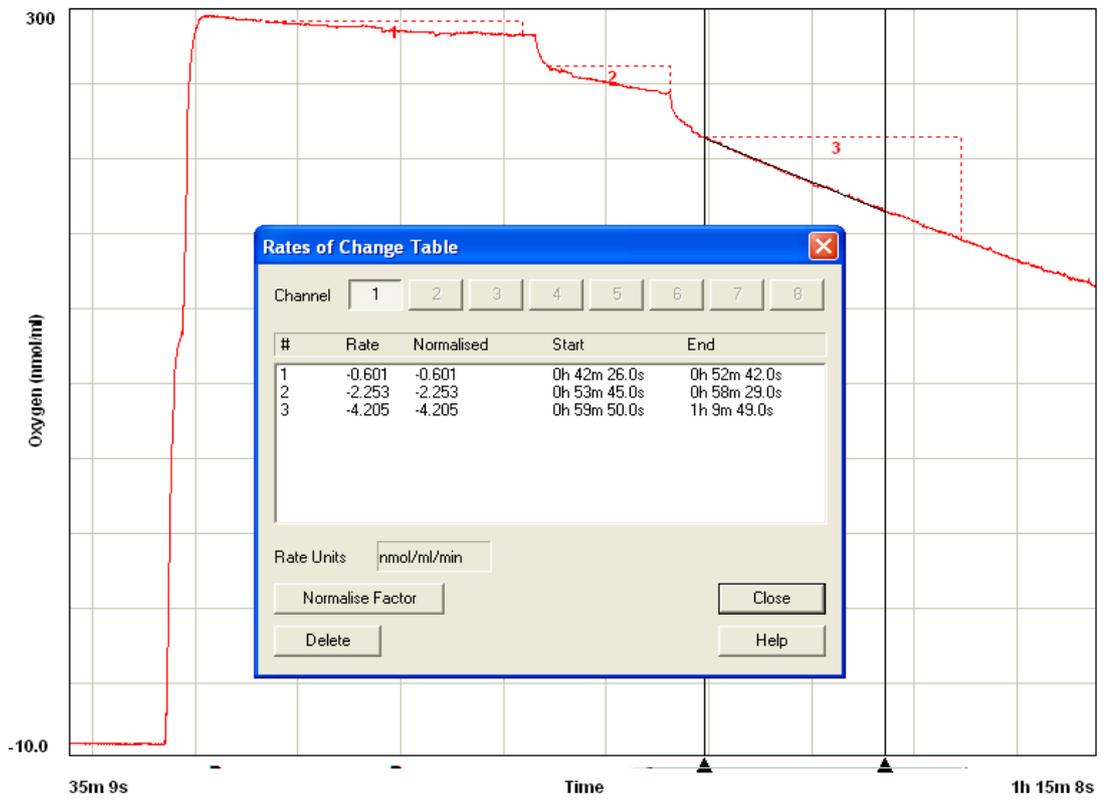
Formula:

Total Biomass = OD * drymass constant * culture volume = **33.89 mg cells**

The N₂O reduction rate (qcN₂O, Table 12) per mg cells can now be determined as followed:

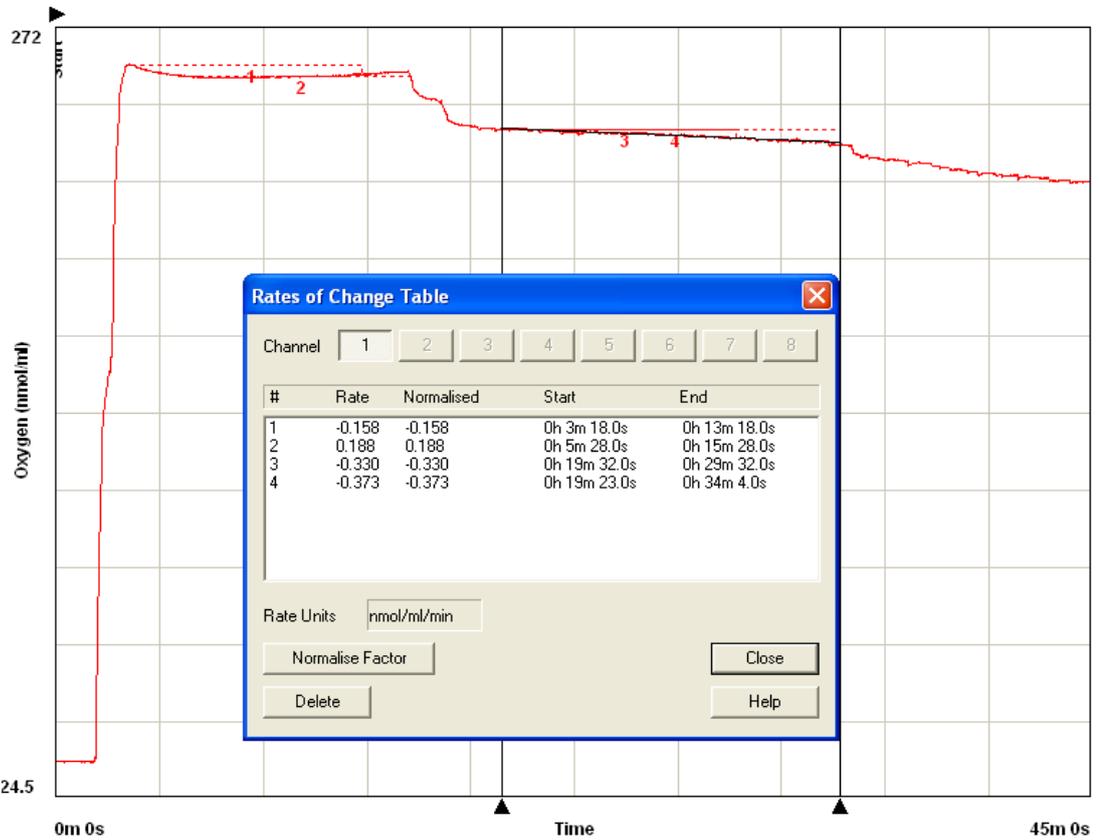
0.0187 [mM * min⁻¹] N₂O / 33.89 = 0.55 nmole * min⁻¹ * mg⁻¹ N₂O

Pd1222 @ 20°C



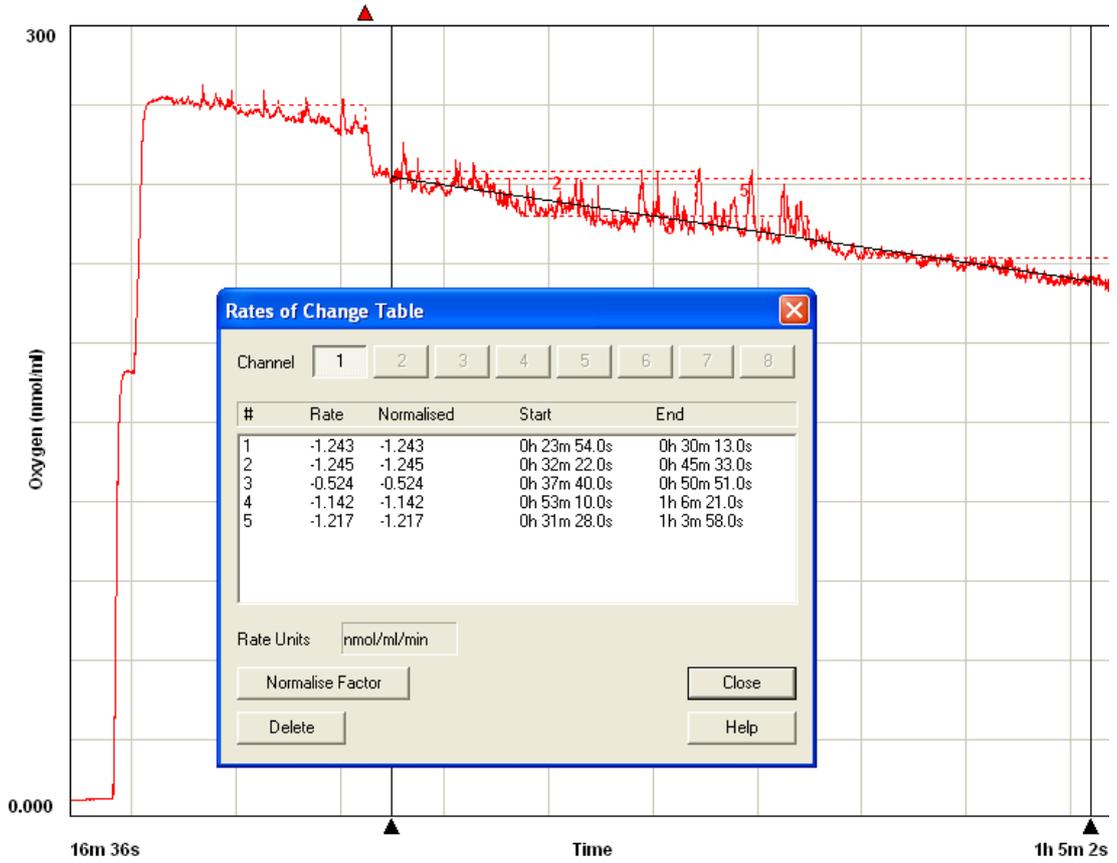
For experimental procedure see Figure 28

SL1344 WT @ 20°C



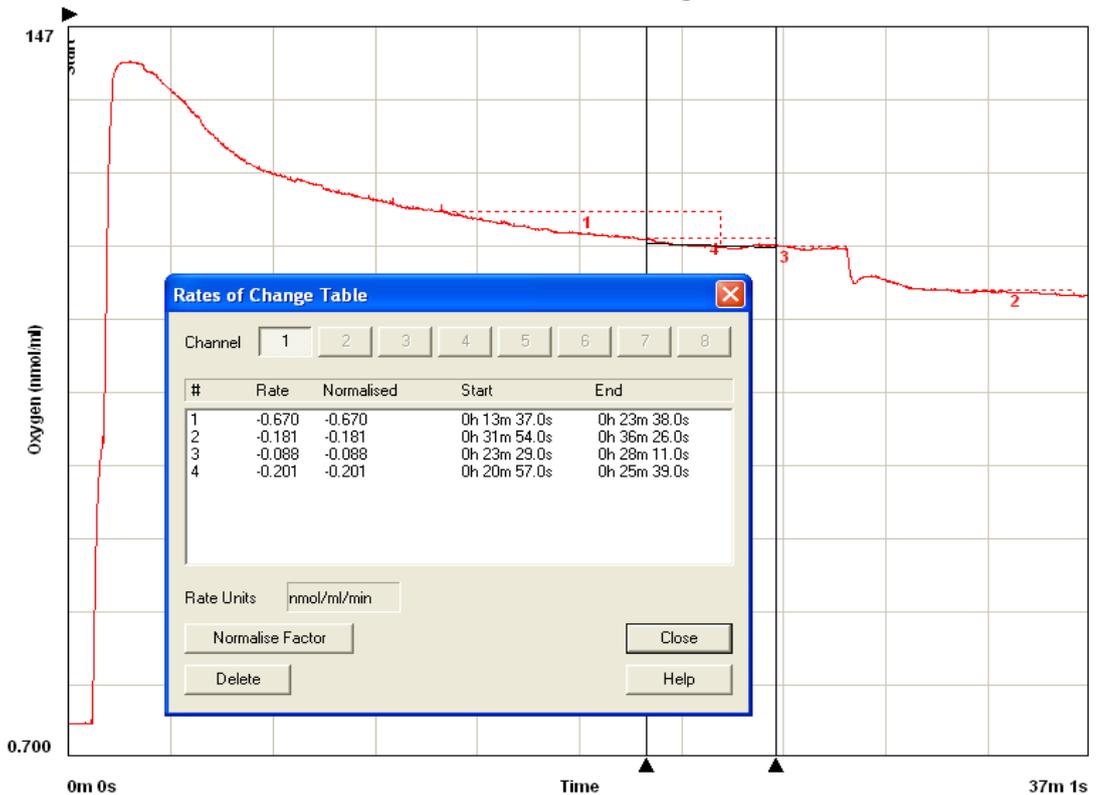
For experimental procedure see Figure 28

SL1344 pH 6 @ 37°C



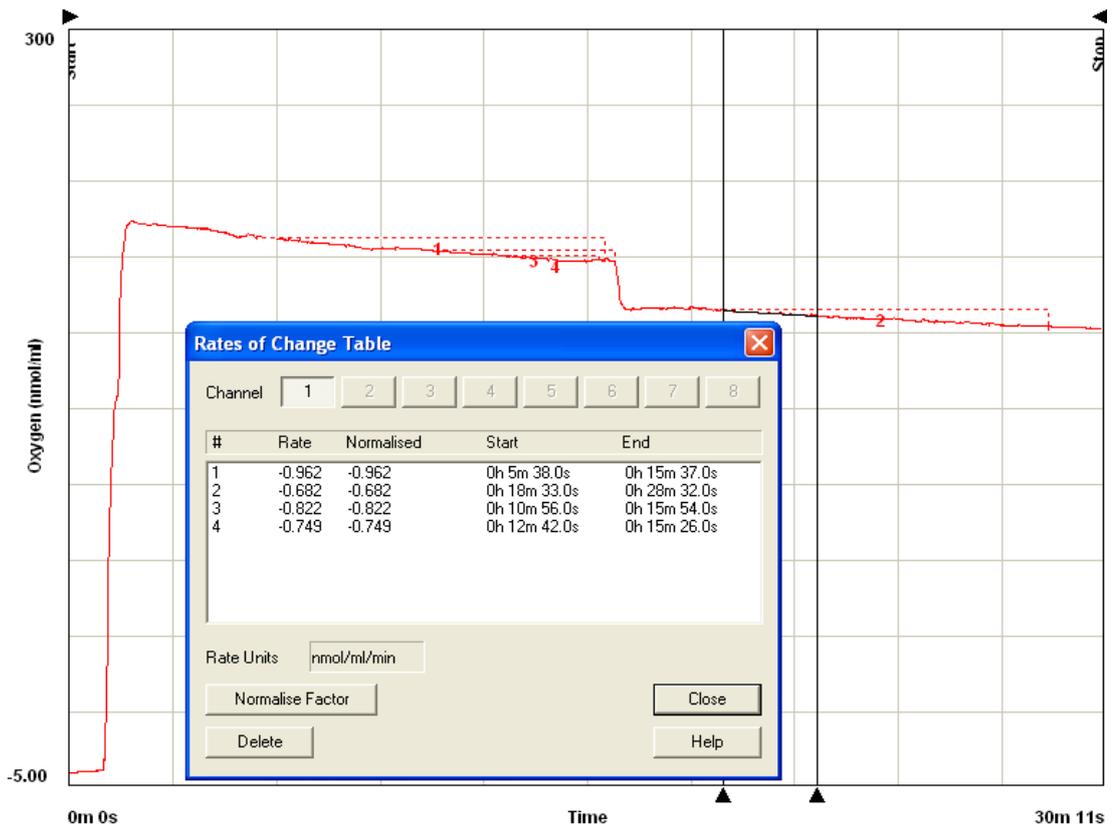
For experimental procedure see Figure 28

MGN G+/N- media @ 37°C



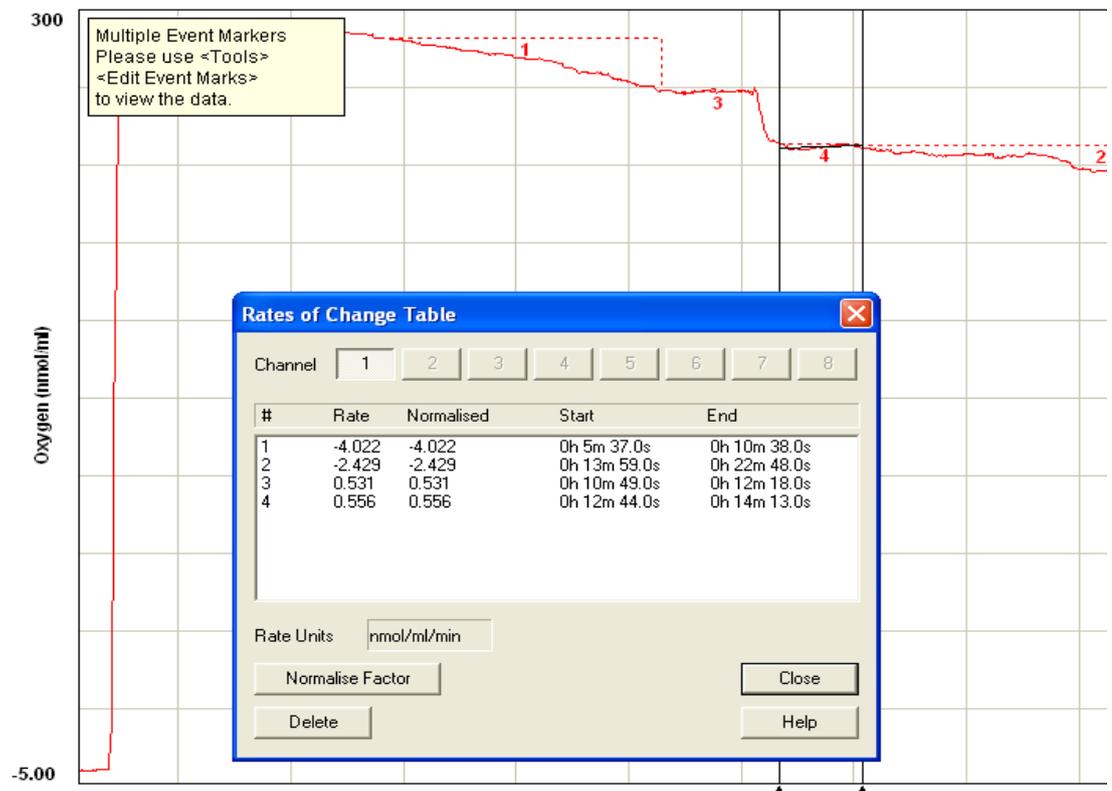
For experimental procedure see Figure 28 (no cells added; negative control)

MGN G+ NO₃⁻ - free media @ 37°C



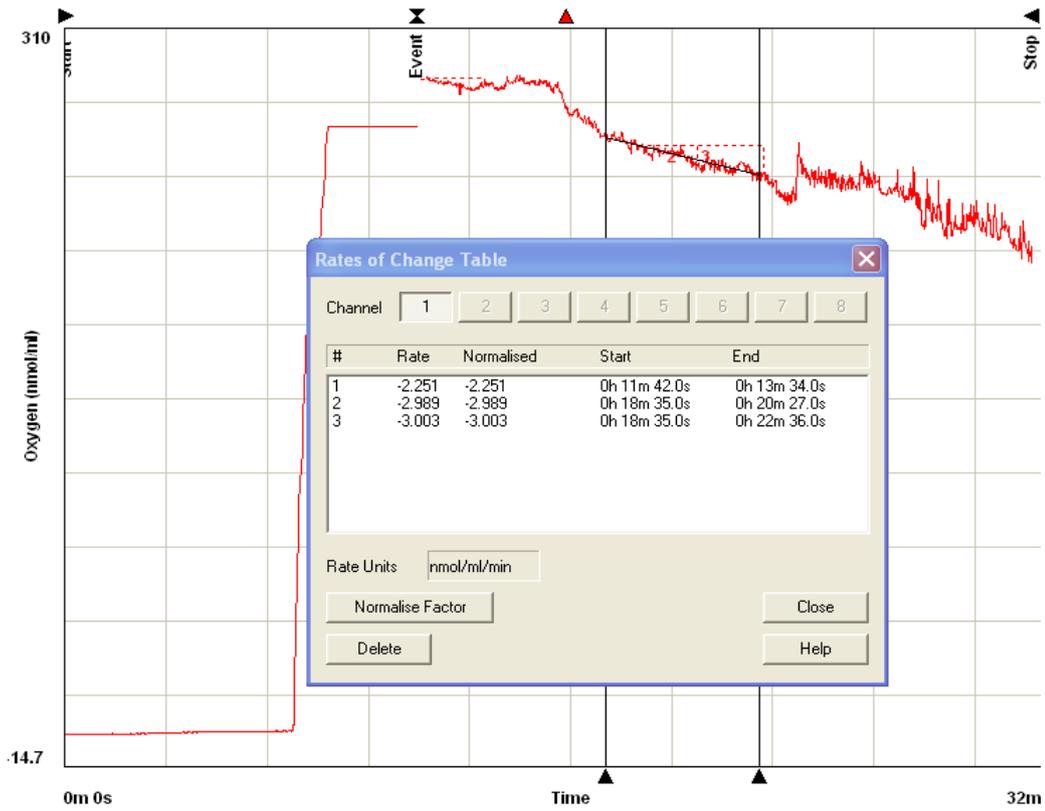
For experimental procedure see Figure 28 (no cells added; negative control)

Dead (autoclaved cells) @ 37°C



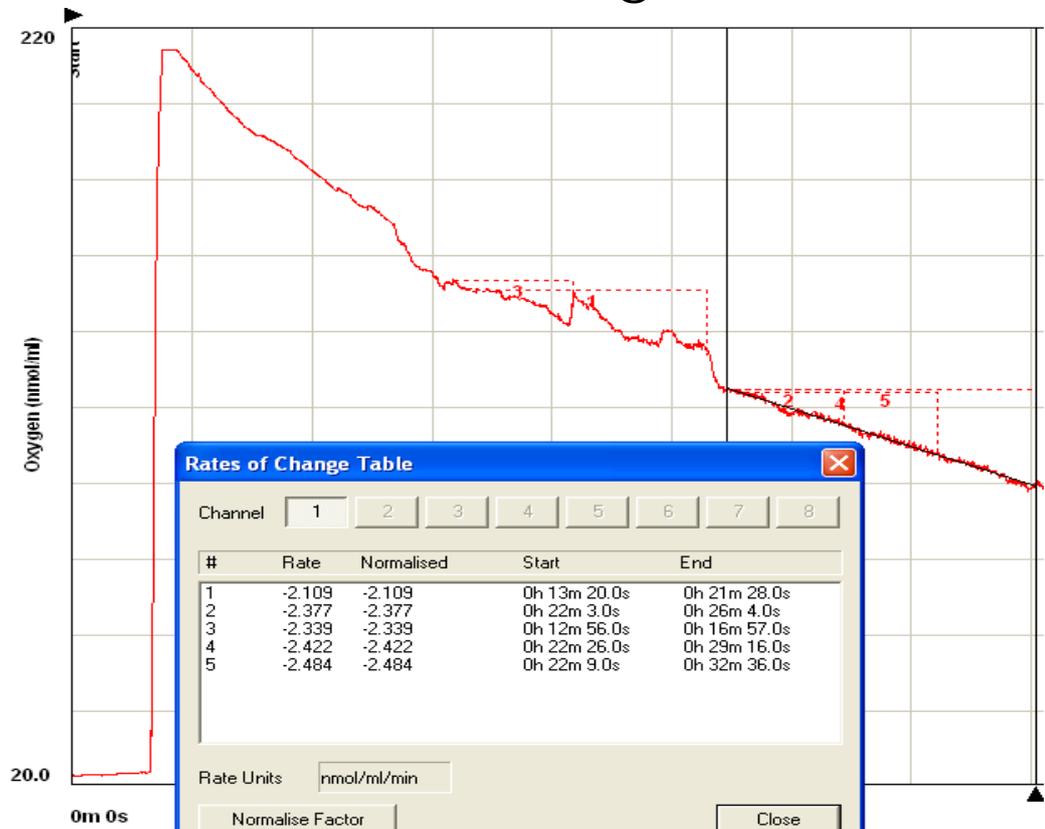
For experimental procedure see Figure 28 (dead cells added; negative control)

SL1344 $\Delta cueO$ @ 37°C



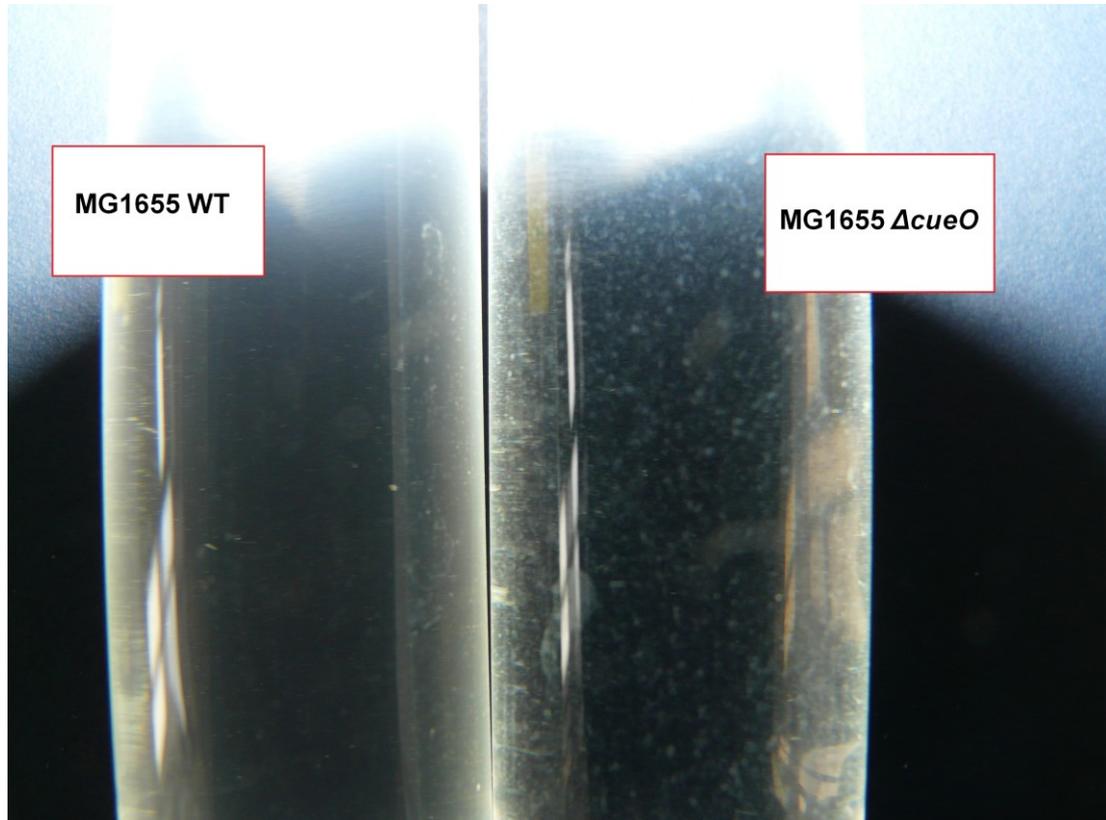
For experimental procedure see Figure 28

SL1344 $\Delta nsrR$ @ 37°C



For experimental procedure see Figure 28

D) Growth in anaerobic Hungate tubes under nitrate-sufficient conditions MG1655 WT vs. $\Delta cueO$



MG1655 WT and $\Delta cueO$ were grown in 10 mL MGN (N^+/G^-) medium, inoculated with 2% of a MGN overnight as described in section 2.7.

E) Microarray table

Appendix E. Genes more than 2-fold higher and lower transcribed in *S. Typhimurium* Δ *nsrR* anaerobic vs. aerobic growth conditions from G⁺/N⁻ chemostat cultures.

Category	Gene	Fold change	Function	
Cytochrome c Biogenesis Up-regulated	<i>ccmA</i>	10.09	cytochrome c biogenesis protein CcmA	
	<i>ccmB</i>	4.59	heme exporter protein	
	<i>ccmC</i>	5.26	heme exporter protein	
	<i>ccmE</i>	5.65	cytochrome c-type biogenesis protein CcmE;heme exporter protein C	
	<i>ccmF</i>	2.14	cytochrome c-type biogenesis protein	
	<i>ccmG</i>	3.06	heme lyase disulfide oxidoreductase	
	<i>ccmH</i>	4.12	putative heme lyase subunit	
	<i>STM3820</i>	3.21	putative cytochrome c peroxidase	
	Nitrate metabolism Up-regulated	<i>glnA</i>	11.09	glutamine synthetase
<i>glnK</i>		3.58	nitrogen regulatory protein P-II 2	
<i>glnL</i>		4.85	nitrogen regulation protein NR(II)	
<i>hcp</i>		12.32	unclear	
<i>hcr</i>		21.05	Hcp oxidoreductase, NADH-dependent	
<i>metR</i>		2.04	metE/metH regulator	
<i>napA</i>		12.30	nitrate reductase catalytic subunit	
<i>napB</i>		9.26	citrate reductase cytochrome c-type subunit	
<i>napC</i>		12.97	cytochrome c-type protein NapC	
<i>napD</i>		5.43	assembly protein for periplasmic nitrate reductase	
<i>napF</i>		5.00	ferredoxin-type protein	
<i>napG</i>		11.56	quinol dehydrogenase periplasmic component	
<i>napH</i>		6.80	quinol dehydrogenase membrane component	
<i>narG</i>		4.81	nitrate reductase 1 alpha subunit	
<i>narH</i>		11.66	nitrate reductase 1 beta subunit	
<i>narI</i>		4.95	nitrate reductase 1 gamma subunit	
<i>narJ</i>		6.49	nitrate reductase 1 delta subunit	
<i>nirB</i>		5.46	nitrite reductase large subunit	
<i>nirC</i>		2.08	nitrite transporter NirC	
<i>nirD</i>		9.01	nitrite reductase small subunit	
<i>nrfA</i>		3.94	cytochrome c552	
<i>nrfB</i>		4.78	cytochrome c nitrite reductase pentaheme subunit	
<i>nrfC</i>		13.26	putative formate-dependent nitrite reductase	
<i>nrfD</i>		2.43	putative formate-dependent nitrate reductase	
<i>nrfE</i>		2.68	formate-dependent nitrite reductase	
<i>soxS</i>		3.97	DNA-binding transcriptional regulator SoxS	
<i>STM4466</i>		2.21	carbamate kinase	
Nitrate metabolism Down-regulated		<i>fur</i>	5.48	ferric uptake regulator
		<i>narU</i>	3.12	nitrate extrusion protein
		<i>narZ</i>	9.87	nitrate reductase 2 alpha subunit
	<i>ogt</i>	6.84	O-6-alkylguanine-DNA:cysteine-protein methyltransferase	
	<i>soxR</i>	2.85	redox-sensing transcriptional activator	
	<i>STM1809</i>	2.54	putative cytoplasmic protein	
	<i>yeaR</i>	3.93	putative cytoplasmic protein	
	<i>ygbA</i>	2.82	hypothetical protein	
	<i>yoaG</i>	4.44	putative cytoplasmic protein	
	<i>ytfE</i>	2.31	iron-sulfur cluster repair di-iron protein	

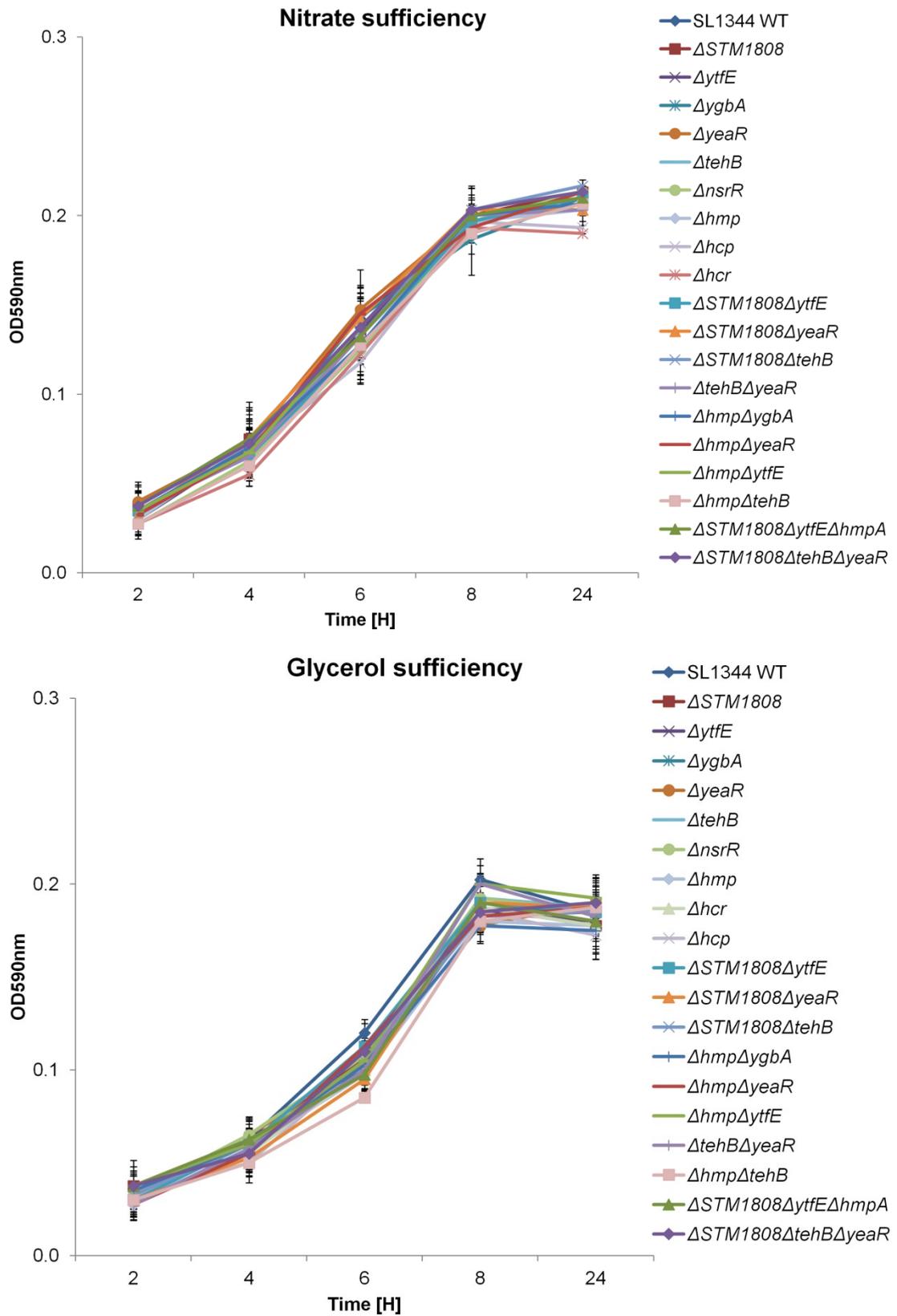
Appendix E cont.

Category	Gene	Fold change	Function
Oxidative stress Up-regulated	<i>ahpC</i>	6.25	alkyl hydroperoxide reductase subunit C
	<i>ahpF</i>	4.48	alkyl hydroperoxide reductase F52a subunit
	<i>katG</i>	5.99	hydroperoxidase
	<i>nmpC</i>	68.49	putative outer membrane porin precursor
	<i>sodB</i>	2.08	superoxide dismutase
	<i>soxS</i>	3.97	DNA-binding transcriptional regulator SoxS
Oxidative stress Down-regulated	<i>arcA</i>	5.05	two-component response regulator
	<i>arcB</i>	2.01	aerobic respiration control sensor protein ArcB
	<i>dksA</i>	3.20	DnaK transcriptional regulator DksA
	<i>dps</i>	4.82	DNA starvation/stationary phase protection protein Dps
	<i>dsbG</i>	2.02	disulfide isomerase/thiol-disulfide oxidase
	<i>fur</i>	5.48	ferric uptake regulator
	<i>grxB</i>	3.74	glutaredoxin 2
	<i>msrA</i>	2.14	methionine sulfoxide reductase A
	<i>sodA</i>	6.30	superoxide dismutase
	<i>sodC</i>	10.81	superoxide dismutase
	<i>soxR</i>	2.85	redox-sensing transcriptional activator
	<i>tpx</i>	4.50	thiol peroxidase
	<i>trxA</i>	2.95	thioredoxin
	<i>trxB</i>	2.02	thioredoxin reductase
sRNA Up-regulated	<i>cyaR</i>	7.14	Unknown
	<i>glmY</i>	2.85	Unknown
	<i>glnL</i>	4.85	nitrogen regulation protein NR(II)
	<i>isrG</i>	2.46	Unknown
	<i>isrL</i>	2.09	Unknown
	<i>PSLT065</i>	2.44	Unknown
	<i>PSLT071</i>	2.58	Unknown
	<i>rydC</i>	3.88	Unknown
	<i>ryeC</i>	2.92	Unknown
	<i>spvR</i>	2.07	Unknown
	<i>sroA</i>	2.38	Unknown
	<i>tpke11</i>	16.58	Unknown
sRNA Down-regulated	<i>dsrA</i>	20.61	Unknown
	<i>isrA</i>	2.51	Unknown
	<i>isrI</i>	3.96	Unknown
	<i>isrJ</i>	3.31	Unknown
	<i>istR</i>	4.30	Unknown
	<i>PSLT026</i>	6.14	putative periplasmic protein
	<i>PSLT047</i>	2.58	putative cytoplasmic protein
	<i>rprA</i>	5.55	Unknown
	<i>rybA</i>	5.22	Unknown
	<i>ryeB</i>	17.88	putative cytoplasmic protein
	<i>rygC</i>	2.79	Unknown
	<i>ryhB-1</i>	2.68	Unknown
	<i>ryhB-2</i>	6.18	Unknown
	<i>sgrS</i>	2.25	Unknown
	<i>spf</i>	43.39	Unknown
	<i>sraA</i>	3.34	Unknown
	<i>sraB</i>	2.53	Unknown
<i>sroC</i>	2.40	Unknown	
<i>tp2</i>	4.80	Unknown	

Appendix E cont.

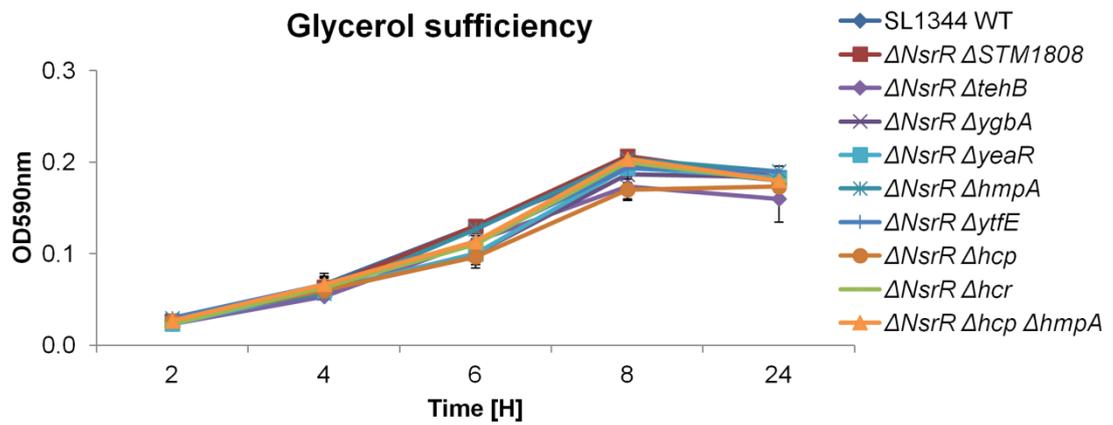
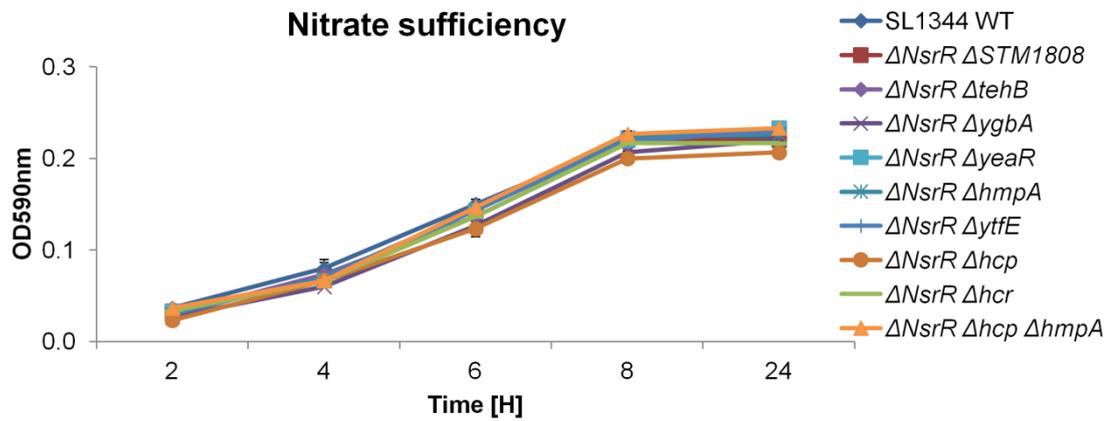
Category	Gene	Fold change	Function	
Vitamin B12 Up-regulated	<i>btuF</i>	2.35	vitamin B12-transporter protein BtuF	
	<i>cbiA</i>	2.53	cobyrinic acid a,c-diamide synthase	
	<i>cbiD</i>	2.02	cobalt-precorrin-6A synthase	
	<i>cbiF</i>	2.25	vitamin B12 biosynthetic protein	
	<i>cbiK</i>	2.07	vitamin B12 biosynthetic protein	
	<i>cbiN</i>	2.48	cobalt transport protein CbiN	
	<i>cobD</i>	2.12	cobalamin biosynthesis protein	
	<i>cysG</i>	3.31	siroheme synthase	
	<i>eutD</i>	2.10	phosphotransacetylase	
	<i>ftn</i>	3.11	ferritin	
	<i>metB</i>	2.80	cystathionine gamma-synthase	
	<i>metF</i>	5.26	5,10-methylenetetrahydrofolate reductase	
	<i>metK</i>	2.99	S-adenosylmethionine synthetase	
	<i>metL</i>	2.55	bifunctional aspartate kinase II/homoserine dehydrogenase II	
	<i>pduA</i>	2.67	polyhedral body protein	
	<i>pduC</i>	3.30	propanediol dehydratase large subunit	
	<i>pduD</i>	3.45	propanediol dehydratase medium subunit	
	<i>pduE</i>	4.27	propanediol dehydratase small subunit	
	<i>pduF</i>	2.08	propanediol diffusion facilitator;Unknown	
	<i>pduG</i>	3.22	propanediol dehydratase reactivation protein	
	<i>pduH</i>	2.91	propanediol dehydratase reactivation protein	
	<i>pduJ</i>	4.27	polyhedral body protein	
	<i>pduK</i>	2.79	polyhedral body protein	
	<i>pduM</i>	2.90	propanediol utilization protein	
	<i>pduN</i>	2.20	polyhedral body protein	
	<i>pduP</i>	2.38	CoA-dependent propionaldehyde dehydrogenase	
	<i>pduS</i>	2.25	polyhedral body protein	
	<i>pduT</i>	2.24	polyhedral body protein	
	<i>pudB</i>	3.55	polyhedral body protein	
	Vitamin B12 Down-regulated	<i>aspC</i>	2.74	aromatic amino acid aminotransferase
		<i>cobC</i>	2.29	alpha ribazole-5'-P phosphatase
<i>csrA</i>		7.07	carbon storage regulator	
<i>cyoE</i>		2.56	protoheme IX farnesyltransferase	
<i>cysE</i>		2.14	serine acetyltransferase	
<i>exbD</i>		2.97	biopolymer transport protein ExbD	
<i>fre</i>		2.24	FMN reductase	
<i>ftnB</i>		11.25	ferritin-like protein	
<i>hemA</i>		2.50	glutamyl-tRNA reductase	
<i>hemL</i>		3.59	glutamate-1-semialdehyde aminotransferase	
<i>luxS</i>		5.75	S-ribosylhomocysteinase	
<i>sdaA</i>		2.79	L-serine deaminase I/L-threonine deaminase I	
<i>sseA</i>		3.00	3-mercaptopyruvate sulfurtransferase	
<i>sseB</i>		2.00	enhanced serine sensitivity protein SseB	
<i>yedO</i>		2.70	D-cysteine desulfhydrase	

F) Growth curves NsrR regulon



NsrR regulon mutant strains were grown in 10 mL MGN (N^+/G^- or N^-/G^+) medium, inoculated with 2% of a MGN overnight as described in section 2.7.

NsrR Background Mutants



NsrR regulon mutant strains were grown in 10 mL MGN (N^+/G^- or N^-/G^+) medium, inoculated with 2% of a MGN overnight as described in section 2.7.