Research in Microbiology 167 (2016) 103–113

www.elsevier.com/locate/resmic

Original article

# A discrete role for FNR in the transcriptional response to moderate changes in oxygen by *Haemophilus influenzae* Rd KW20

Donald Jiang [a,b,d], Alexandra Tikhomirova [a,b], Stephen J. Bent [b,c], Stephen P. Kidd [a,b,*]

[a] *Research Centre for Infectious Disease, The University of Adelaide, Adelaide, Australia*
[b] *School of Biological Science, The University of Adelaide, Adelaide, Australia*
[c] *Robinson Research Institute, The University of Adelaide, Adelaide, Australia*
[d] *Agri-Food and Veterinary Authority of Singapore, Singapore*

Received 10 July 2015; accepted 29 September 2015
Available online 21 October 2015

**Abstract**

The survival by pathogenic bacteria within the specific conditions of an anatomical niche is critical for their persistence. These conditions include the combination of toxic chemicals, such as reactive oxygen (ROS) and reactive nitrogen species (RNS), with factors relevant to cell growth, such as oxygen. *Haemophilus influenzae* senses oxygen levels largely through the redox state of the intracellular fumarate-nitrate global regulator (FNR). *H. influenzae* certainly encounters oxygen levels that fluctuate, but in reality, these would rarely reach a state that results in FNR being fully reduced or oxidized. We were therefore interested in the response of *H. influenzae* to ROS and RNS at moderately high or low oxygen levels and the corresponding role of FNR. At these levels of oxygen, even though the growth rate of an *H. influenzae fnr* mutant was similar to wild type, its ROS and RNS tolerance was significantly different. Additionally, the subtle changes in oxygen did alter the whole cell transcriptional profile and this was different between the wild type and *fnr* mutant strains. It was the changed whole cell profile that impacted on ROS/RNS defence, but surprisingly, the FNR-regulated, anaerobic nitrite reductase (NrfA) continued to be expressed and had a role in this phenotype.


*Keywords:* FNR; Aerobic/anaerobic; *Haemophilus influenza*; Oxidative stress

## 1. Introduction

*Haemophilus influenzae* is associated with a range of diseases as a consequence of its infection of several distinct anatomical niches [1]. It is a human host-adapted bacterium and largely remains a commensal of nasopharyngeal mucosa. It can transit from this site of colonization to other parts of the respiratory tract (such as to the middle ear to cause otitis media (OM) or to the lung to cause pneumonia or exacerbations of chronic obstructive pulmonary disease) or systemically (the blood and brain) and this transit results in the various manifestations of disease. The variations between strains and isolates of *H. influenzae* influence their capacity to occupy a site of infection, affecting disease outcome. Capsular strains traditionally have been associated with systemic infections and the non-typeable (NT*Hi*) strains are linked to respiratory tract infection. Although the incidence of *H. influenzae* causing meningitis has been greatly reduced through the introduction of the vaccine against the major serotype (type b) that caused this disease, infection and disease resulting from NT*Hi* continue to have a major impact on human health [2].

The combination of physical and chemical properties within an environment determines the physiological capacity of a bacterium to survive in that environment. These environmental factors include the broad features such as carbon

* Corresponding author. Research Centre for Infectious Disease, The University of Adelaide, Adelaide, Australia.
*E-mail addresses:* Donald_JIANG@ava.gov.sg (D. Jiang), alexandra.tikhomirova@adelaide.edu.au (A. Tikhomirova), Stephen.bent@adelaide.edu.au (S.J. Bent), stephen.kidd@adelaide.edu.au (S.P. Kidd).

http://dx.doi.org/10.1016/j.resmic.2015.09.008
0923-2508/

and energy source, pH, and temperature; but these also interact with the bioavailability of micronutrients and essential elements such as iron, copper and zinc. There is also an interaction between these factors and the toxicity of reactive chemicals that would be present in the host-pathogen environment, such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive aldehydes. As an example, it has been shown that if there are changes in the oxygen tension or pH within human tissue, there is alteration in the toxicity of the ROS or RNS that are present [3,4].

Between anatomical niches relevant to *H. influenzae* colonization and infection, such as the nasopharynx, middle ear and lung, there are known to be differences between physical and chemical properties (such as pH and oxygen). In particular, the oxygen availability has been clearly established as an environmental factor that significantly alters and influences the pathogenesis of *H. influenzae*. In the lung, the environment is mostly aerobic, while the middle ear is anaerobic. Indeed, even within a niche, some of these properties would vary in concentration over time.

In various studies on the bacterial response to changing oxygen levels, it has been established that there are key signal pathways and physiological changes [5–8]. In the model system for facultative anaerobic bacteria, *Escherichia coli*, there are two pathways for sensing oxygen levels. These are the two-component system ArcAB (anoxic redox control) and the intracellular, global regulator FNR (fumarate nitrate regulator). FNR has an iron-sulphur cluster that exists in a [4Fe–4S] dimer state in anaerobic conditions and then, when fully oxidized, becomes an apo-FNR monomer which does not bind DNA. FNR is present in many bacteria, and in some studies it has been shown that FNR can be functional in the transition states between its oxidized and reduced states [5]. Both ArcAB and FNR exist in *H. influenzae* and would appear to broadly function in the same manner as for *E. coli* [9,10]. Previous studies have largely focussed on the regulation and cellular role of these regulatory systems at the extremes of oxygen levels, in particular, in anaerobic conditions where both systems are suggested to be "active" [9,10]. A key study on ArcA in *E. coli* showed significant transcriptional variations as oxygen levels were subtly changed [11]. In a clinical setting, it is likely that *H. influenzae* is equally required to adapt to levels of oxygen that are moderately high and then low (but not anaerobic). These levels would not result in absolutely oxidized or reduced FNR; but in different forms of its transition states it could still be functional, although this would differ from the binary FNR conformation states. This means that, depending on the level of oxygen, there possibly would be a particular transition state of FNR that could bind to a discrete set of its operator/promoter sites and thereby have a particular cellular role. This condition-specific profile of regulated genes could have broad consequences for cellular survival within environmental conditions. In this context, we were interested in investigating the response to ROS and RNS by *H. influenzae* in conditions of moderately high and low oxygen and correspondingly, determining the role of FNR within *H. influenzae* in these conditions.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

For cloning experiments *E. coli* DH5α was used. *E. coli* was grown in Luria–Bertani media. *H. influenzae* was cultured in BHI media which was prepared with 3.7% w/v BHI powder (Oxoid). For solid medium, 1.5% agar powder was added. Medium was sterilized by autoclaving at 121 °C for 20 min. 10% w/v Levinthal blood was added for solid BHI media. BHI broth required $NAD^+$ (2 μg/ml) and 10 μl/ml Hemin solution (0.1% w/v hemin, 0.1% w/v L-histidine, 4% v/v tri-ethanolamine). M-IV competence medium was prepared as previously described [12]. For monitoring cell growth over a time course, *H. influenzae* strains were initially pre-cultured overnight in 5 ml BHI. The $OD_{600nm}$ was measured and a normalized amount of cells inoculated into 50 ml of BHI broth. The cells were grown aerobically by shaking at 200 rpm, at 37 °C. $OD_{600nm}$ measurements were taken at given time-points and assays were performed in triplicate. Growth rates were calculated by assessing the start of the exponential phase of growth (OD at this time, $N_0$) and the end of this phase of growth (OD at this time, $N_t$); and the growth rate ($k$) was $(\log_2 N_t - \log_2 N_0)/t$ (doubling per hour). For analysis of the influence of oxygen supply to the cells, cultures were grown in 250 ml conical flasks with 25 ml, 75 ml and 150 ml medium. This has been previously used and shown to provide the oxygen transfer coefficient (kLa) values of 87.4 $h^{-1}$ (high), 27.8 $h^{-1}$ (medium) and 11.5 $h^{-1}$ (low) respectively [13,14]. For the killing assays, fresh overnight cultures of the particular strains were inoculated and grown for 2 h, the cell numbers assessed by OD (and for verification, by cell counts) and equal numbers inoculated into broth culture containing the calculated concentration of the chemical stress (GSNO, 0.5 mM (Sigma–Aldrich), $H_2O_2$, 2 mM (Sigma–Aldrich) and for nitric oxide, diethylamine-NONOate, 0.1 mM (Sapphire Bioscience)).

### 2.2. Chromosomal inactivation of fnr and nrfA

A 2.0 kb DNA fragment containing *fnr* and 500 bp flanking regions was amplified by PCR from *H. influenzae* using primers:

HIfnrKO-F (5′GTATCAGGTTGCAATCCTTGT) and HIfnrKO-R (5′GAGTAAAGATTAGAGACGTAAG) and the following PCR conditions: 96 °C for 5 min, then 30 cycles of 96 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min and then 72 °C for 5 min using Vent$^R$ Polymerase (New England Biolabs). The PCR product was purified (QIAquick PCR Purification Kit, QIAGEN) and cloned into pT7Blue vector (Perfectly Blunt Cloning Kits, Novagen). The resulting plasmid, pT7Blue::*fnr*KO, was digested with *Bsm*I and the overhangs were removed with Mung bean nuclease (New England Biolabs).

The kanamycin-resistant gene cassette from pUC4Kan was isolated by digestion with *Hinc*II and extracted from 1% agarose gel using a QIAquick gel extraction kit (QIAGEN). This cassette