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Original article

Haemophilus influenzae strains possess variations in the global transcriptional profile in response to oxygen levels and this influences sensitivity to environmental stresses

Donald Jiang[1], Alexandra Tikhomirova, Stephen P. Kidd*

Research Centre for Infectious Diseases, School of Molecular and Biomedical Sciences, The University of Adelaide, North Terrace Campus, Adelaide, South Australia, 5005, Australia

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Abstract

An alcohol dehydrogenase, AdhC, is required for *Haemophilus influenzae* Rd KW20 growth with high oxygen. AdhC protects against both exogenous and metabolically generated, endogenous reactive aldehydes. However, *adhC* in the strain 86-028NP is a pseudogene. Unlike the Rd KW20 *adhC* mutant, 86-028NP does grow with high oxygen. This suggests the differences between Rd KW20 and 86-028NP include broader pathways, such as for the maintenance of redox and metabolism that avoids the toxicity related to oxygen. We hypothesized that these differences affect their resistance to relevant toxic chemicals, including reactive aldehydes. Across a range of oxygen concentrations, despite the growth profiles of Rd KW20 and 86-028NP being similar, there was a significant variation in their sensitivity to reactive aldehydes. 86-028NP is more sensitive to methylglyoxal, formaldehyde and glycolaldehyde under high oxygen than low oxygen as well as compared to Rd KW20. Also, as oxygen levels changed the whole genome gene expression profiles of Rd KW20 and 86-028NP revealed distinctions in their transcriptomes (the iron, FNR and ArcAB regulons). These were indicative of a difference in their intracellular redox properties and we show it is this that underpins their survival against reactive aldehydes.

Keywords: *Haemophilus influenzae*; Oxygen metabolism; Oxidative stress; Metabolic resistance; Stress response; Reactive aldehydes

1. Introduction

Haemophilus influenzae colonizes the human nasopharynx and mostly remains as a commensal at this site of the body. Some strains have an ability to migrate from the nasopharynx to other parts of the body and in doing this they can cause numerous types of disease [1]. This ability involves colonising further organs and tissues that are known to vary in physical and chemical parameters. These include: oxygen tension, carbon/energy/nitrogen source, pH, varying levels of toxic reactive oxygen and reactive nitrogen species (ROS and RNS, respectively) and the availability of essential metal ions (especially iron but also copper, zinc and manganese) [2–9].

A difficult but important consideration when studying the bacterial response to any of these environmental factors is that they exist in combination. As an example, the toxicity of ROS and RNS is influenced by the pH and/or the oxygen levels [10–12]. Likewise in aerobic conditions there is an increase in the bacterial requirement for iron (for energy production), the exact conditions that increase its toxicity (through Fenton chemistry). An additional example is that growth under aerobic conditions can be driven through the tricarboxylic acid

* Corresponding author. Tel.: +61 08 83134671; fax: +61 08 83134362.
E-mail address: stephen.kidd@adelaide.edu.au (S.P. Kidd).
[1] Present address: Agri-Food and Veterinary Authority of Singapore, 10 Perahu Road, Singapore 718837, Singapore.

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cycle (TCA cycle) and the Pentose Phosphate Pathway (PPP) for the essential production of substrates for biosynthesis of biomolecules, cofactors and maintaining the intracellular conditions such as the redox potential (this is exactly the case for *H. influenzae* [13]). Some of the intermediates of these two pathways (such as glyceraldehyde-3-phosphate and erythrose-4-phosphate) are short chain aldehydes/sugars. Short chain aldose sugars under these aerobic conditions undergo auto-oxidation to toxic dicarbonyl species (reactive aldehydes) [14–16]. Clearly the study of the bacterial response to one environmental stress must be conscious of other existing and impacting factors.

We have previously reported that *H. influenzae* Rd KW20 possesses a novel response to both endogenously generated as well as exogenous reactive aldehydes. This is driven by the NmlR transcription factor and its regulatory target; *adhC-estD* (an alcohol dehydrogenase and esterase D operon) [17,18]. AdhC is present in various bacteria and is multi-functional; defending against the RNS *S*-nitrosoglutathione (GSNO) as well as endogenous toxic by-products of the cell's own metabolism (formaldehyde and other aldehydes). We found that the *adhC* expression was essential in the *H. influenzae* strain Rd KW20 during aerobic growth, as a response to reactive aldehydes [17]. Interestingly, there are variations in the genetics of *nmlR-adhC* across clinical isolates. Most significantly we found *adhC* in the non-typeable (NTHi) strain 86-028NP was a pseudogene [18].

There are differences in the iron requirement between isolates of *H. influenzae* [19–22]. *H. influenzae* does not possess the complete biosynthetic pathways for produce protoporphyrin IX (PPIX) and therefore relies on acquiring heme from the environment (or iron and PPIX) [23–25]. Studies have identified the global effects of iron, the core modulon linked to iron/heme regulated genes and the gene expression of the iron/heme responsive genes *in vivo* [20,21]. Interestingly, and more so than several iron-bound, iron-storage or iron-transport genes, in conditions of added iron, *adhC* was one of the highest of the up-regulated genes [22] and this was replicated across strains [20,21]. Separate studies have shown *adhC* to be highly important in pathways for the bacterial response to oxygen levels, reactive aldehydes, and iron metabolism and linked to central metabolism during aerobic growth and this has an interplay with iron. AdhC seems to be at the cross section of these pathways. Despite this central role, in 86-028NP *adhC* is non-functional (although it grows with oxygen). There have been focussed studies on specific iron-associated molecular pathways in 86-028NP, such as into FUR [26], heme utilization and transport [27] and environmental sensing of appropriate iron (ferrous iron, by FirRS) [28]. There are obviously pathways that overlap with iron-uptake, and utilization, these are linked to core metabolic processes as well as stress response (such as for ROS) and they vary between the Rd KW20 and 86-028NP isolates of *H. influenzae*. We were interested in determining the difference in the iron-dependent growth and oxygen levels and reactive aldehyde sensitivity of 86-028NP compared to Rd KW20.

2. Materials and methods

2.1. Bacterial strains and growth conditions

H. influenzae was cultured on brain heart infusion (BHI) medium or chemically defined media (CDM). BHI was prepared with 3.7% (wt/vol) BHI Powder (Oxoid). For solid medium, 1.5% (wt/vol) agar powder was added. Media were sterilized by autoclaving at 121 °C for 20 min. Levinthal blood (10% [wt/vol]) was added for solid medium; NAD (2 μg/ml) and 10 μg/ml hemin solution (0.1% [wt/vol] hemin, 0.1% [wt/vol] L-histidine, 4% [vol/vol] triethanolamine) was added to broth. CDM was prepared as described by Coleman et al. [29].

2.2. Growth assays

The growth analysis of *H. influenzae* was carried out using CDM. For analysis of the effects of oxygen supply to the cells, cultures were grown in 250 ml conical flasks with varying volumes of broth added; 25 ml for high oxygen or 150 ml for low oxygen (and 220 rpm or 100 rpm shaking, respectively). These parameters have been shown to provide the oxygen transfer coefficents (kLa) values of 87.4 h^{-1} (high) and 11.5 h^{-1} (low) respectively [30,31].

Different concentrations (Fig. S1) of the reactive aldehyde agents were added to the medium. When cells were at the end of logarithmic phase, 50 μl was taken and serially diluted (1/100) in PBS and 20 μl plated onto BHI agar plates. Cells were enumerated by the colony forming units (CFU)/ml being calculated. Each assay was done in triplicate, the experiment was repeated as three biological replicates (statistical analysis was performed using two-tailed Student t-test).

Growth with limiting iron was achieved using the iron-specific chelator (desferrioxamine meslyate, DAM). Initial experiments were carried out with increasing DAM concentrations (up to 1.5 mM) to determine the concentration that limited the iron levels to the point of preventing growth. Hemin was added to permit growth.

Growth with glutathione as a means of restoring and maintaining the redox balance in *H. influenzae* during reactive aldehyde stress was achieved as described above for the reactive aldehyde assays but concurrently glutathione (50 μM) and DTT (250 μM) was added in these assays and the growth recorded.

2.3. Transcriptomics studies

We have previously performed RNAseq analysis on Rd KW20 under high and low oxygen (manuscript under review, this data is added in here as a comparison). In exactly the same methodology, 86-028NP cells were cultured under the growth conditions as we defined above as high and low oxygen (OD_{600} ~0.3, determined as the early log phase) and cells collected for RNA extraction; to prevent RNA from degradation [32] the cells were sampled directly into a phenol/ethanol solution (5% v/v phenol (pH 4.3), 95% v/v ethanol). The ratio used is 2/5 of the total cell culture volume:phenol/ethanol.