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Detecting virulence and drug resistance mycobacterial phenotypes *in vivo*

Graham Timmins

Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico Health Sciences Center, Albuquerque NM 87131 USA

Abstract

Bacterial phenotypes are predominantly studied in culture because detection of their specific metabolic pathways in the host is challenging. Development of stable isotope breath tests allowing *in situ* phenotype analyses may endow diagnostics with new modalities based upon direct monitoring of *in vivo* microbial metabolism and host–pathogen phenotypic interactions.

Keywords

Bacteria; breath test; diagnosis; metabolism; phenotype

Bacterial phenotypes in the host

Mycobacterium tuberculosis is a successful human pathogen because of two key attributes: (i) it uses its wide range of metabolic pathways to grow, survive, or persist in greatly differing cellular and anatomic sites of infection, and; (ii) it is able to produce a plethora of molecules that defend against and selectively control the host immune response. These mycobacterial phenotypes determine the key host–pathogen interactions, and also control drug resistance. Strong associations between genotype and phenotype frequently allow the use of genetic information to reliably predict and diagnose a phenotype (*e.g.* diagnosis of tuberculosis with rifampin sensitivity through Xpert MTB/RIF). However, clinically important cases exist where the genotype to phenotype linkage is weak or non-existent. For example, although mutations in *pncA* drive pyrazinamide resistance in *M. tuberculosis*, the linkage between many known mutations with resistance is difficult to establish and monitor in a clinical setting [1]. Furthermore, many virulence-associated phenotypes such as quorum sensing or biofilm growth cannot be determined from DNA sequence, and although RNA sequencing can enable linkage of phenotypes with their transcriptomes, the use of such

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Corresponding author: Timmins, G. (gtimmins@salud.unm.edu).

Disclaimer Statement

I am co-founder and Chief Science Officer of Avisa Pharma, a company that licenses UNM patents of which I am an inventor. Avisa Pharma is developing an inhaled ¹³C-urea breath test for diagnosing serious lung infections.

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approaches in clinical infectious disease diagnosis poses many challenges and awaits practical development [2].

Classically, many established medical microbiology tests use the phenotypes of cultures isolated from patients to identify species, or their antibiotic sensitivities through growth and biochemical surrogates such as metabolism. However, obtaining culture samples can be challenging either due to difficulties in defining or accessing the infection site, due to the sampled bacteria being in viable but nonculturable states, requiring fastidious culture conditions, or due to slow growth. Additionally due to profound differences between the hostile host environment and the permissive culture environment, phenotypes important in the host–pathogen interface may not be expressed in culture. Therefore, insights from bacterial culture may not mimic what occurs in the host, and so important phenotypes such as drug resistance need to be studied *in situ* in the host to gain insights into the host–pathogen interface, and to enable rapid diagnosis of bacterial presence, identity, or drug sensitivity.

Stable isotope tracers for investigating *in situ* phenotypes

The idea is simple: stable isotope tracers can undergo specific bacterial metabolism and the labeled products (*e.g.* in breath) are analyzed to indicate bacterial metabolic phenotypes. Their enzymatic nature enables rapid amplification of the signal to detectable levels. The archetype of this approach is the ^{13}C -urea breath test for *Helicobacter pylori* in which a labeled urea drink specifically delivered to the infection site (stomach) where *H. pylori* urease, a key virulence factor, converts the ^{13}C -urea to $^{13}\text{CO}_2$, which is excreted in breath. Although intestinal urease-expressing bacteria exist as potential confounders, there is a ‘kinetic window’ of gastric specificity early after urea delivery (before it enters the intestines) that enables impressive specificity. Furthermore, the effectiveness of *H. pylori* eradication therapy can be confirmed by a negative urea breath test after antibiotic therapy.

Lung pathogens could, in theory, be detected and studied in a similar manner by breath tests. Delivery by either nebulization or dry-powder inhalation would enable specific sampling of lungs, with early sampling of exhaled breath precluding contributions by the complex gut microbiome. *M. tuberculosis* was tested as this is a bacterial pathogen that expresses the virulence factor urease [3, 4], an enzyme traditionally used in phenotypic culture identification [5]. Direct lung delivery in a rabbit model of tuberculosis was used and discrimination between infection and controls was possible soon after delivery [6]. Additionally, $^{13}\text{CO}_2$ levels correlated with bacterial burden during antibiotic treatment and withdrawal, suggesting that the initial efficacy of a regimen (and hence drug sensitivity) might be rapidly and simply determined in a manner similar to early bactericidal activity studies [6]. However, urease is widely expressed by lung pathogens, and although it broadly allows bacterial presence and response to therapy measurements, identification of specific species requires additional approaches.

This led to study of more specific pathways to enable higher confidence in species identity determination. One pathway that appeared very specific for mycobacteria was CO dehydrogenase (CODH) that oxidizes CO to CO_2 . Furthermore, CO has shown powerful

effects upon mycobacterial gene expression and phenotype [7, 8], and so CODH might be important in the host–pathogen interface by controlling CO levels. Use of doubly-labeled $^{13}\text{C}^{18}\text{O}$ that was converted by CODH to $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ enabled effective discrimination between infected and control animals [9]. Oxygen exchange by carbonic anhydrase required suppression through pharmacological inhibition to retain significant $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ signal. However, clinical use of this technique will probably be limited by the CO dose required. Although use of a microdose of ^{14}CO and detection of $^{14}\text{CO}_2$ by accelerator mass spectrometry would overcome these limitations, deployment is unlikely in resource-limited settings.

A key clinical phenotype is drug sensitivity, and many key antibiotics for tuberculosis are prodrugs that are converted by mycobacterial enzymes to active forms. Mutations in genes coding for activating enzyme systems that prevent prodrug activation frequently result in high levels of resistance. The isotope breath test could be used to detect prodrug activation because this involves oxidative or hydrolytic mechanisms resulting in the formation of volatile species that can be detected in breath. In the case of isoniazid (INH), oxidative activation by mycobacterial KatG results in formation of an isonicotinylacyl radical that forms adducts with NAD(H) and NAD(P)H, the former being potent inhibitors of mycolic acid synthesis [10]. Formation of this radical is thought to involve beta cleavage of a precursor hydrazyl radical and release of $\text{HN}=\text{NH}$, diazene, that can reduce double bonds or disproportionate to form N_2 [11, 12]. By labeling the INH hydrazide with ^{15}N , detection of KatG activation of INH by the resulting $^{15}\text{N}_2$ product was sought. *In vitro* studies showed species specificity compared to some common lung pathogens, and it was possible to discriminate between a drug sensitive and a resistant S315T KatG mutant strain that shows decreased INH activation [11]. Studies in the rabbit model showed the ability to discriminate between infected and control animals rapidly (within 5 minutes after lung delivery), and appeared to show a dependence of $^{15}\text{N}_2$ signal magnitude upon bacterial load [11]. In all cases, only active disease was studied, and it is unclear what lower bacterial burdens (such as in latent or pre-clinical disease) these techniques might be sensitive enough to detect subclinical or asymptomatic infections. Similarly, mixed resistant/sensitive populations might prove difficult unless one dominates, as will occur for the resistant phenotype upon treatment.

The preclinical development of other tuberculosis prodrug breath tests is ongoing, and may help to prevent resistance in drug regimens such as PaMZ (pretomanid moxifloxacin and pyrazinamide). Other non-tuberculosis projects underway include breath test detection of lung *Francisella tularensis* infection through conversion of ^{13}C -citrulline to $^{13}\text{CO}_2$ by the virulence factor citrulline ureidase, and detection of *Clostridium difficile* through $^{13}\text{CO}_2$ released during *p*-cresol production.

Concluding remarks

It is an exciting development that by using stable isotope breath tests we can detect bacterial phenotypic properties including critical drug sensitivity *in situ*. The lessons learned (Box 1) will help to more generally apply this approach, in order to broadly understand the complex

and varied roles of bacterial metabolic phenotypes that control both host and bacteria homeostasis in asymptomatic states and overt infectious disease.

Box 1

Key lessons learned from stable isotope tracer studies

1. **Delivery:** Specific tracer delivery and establishment of ‘kinetic window’ parameters (during which only certain organ systems are sampled) enables study of key sites while preventing interference from other sites such as the gut microbiome.
2. **Specificity:** *In silico* searches for specific metabolic pathways need experimental verification using other common bacteria for that site. There also has to be some degree of bacterial specificity, as if the host performs the same transformation it will be difficult to impossible to attribute signal detection to a bacterial source. Thus many pathways used in *in vitro* speciation of isolated bacteria (glucose oxidation/fermentation or catalase) are not suitable for this approach.
3. **Detectability:** Dilution of bacterial signal in the analyte to be sampled need consideration. In breath, bacterial $^{13}\text{CO}_2$ signals are diluted by exhaled host CO_2 and may not be detected against this large background. However, isotopomers that are rare in breath (such as $^{15}\text{N}_2$), give bacterial signals resolvable even against large breath backgrounds. Alternatively, the detection of bacteria-specific isotopomers of species that are chemically rarer in breath (*e.g.* ^{15}NO) undergo less dilution with endogenous exhaled gas and enabling sensitive detection.
4. **Detector portability:** Some species like $^{13}\text{CO}_2$ can be detected by infrared spectroscopy enabling point of care (POC) diagnostic approaches. Others require mass spectrometry so that POC use requires development.
5. **Collaboration:** Isotope ratio mass spectrometry capabilities for breath analysis are often found in geoscience departments, while isotope tracer synthesis may need a chemist, and animal models are available to biologists: multidisciplinary teams are therefore needed.

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