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Exometabolomics and MSI: deconstructing how cells interact to transform their small molecule environment

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Metabolism is at the heart of many biotechnologies from biofuels to medical diagnostics. Metabolomic methods that provide glimpses into cellular metabolism have rapidly developed into a critical component of the biotechnological development process. Most metabolomics methods have focused on what is happening inside the cell. Equally important are the biochemical transformations of the cell, and their effect on other cells and their environment; the exometabolome. Exometabolomics is therefore gaining popularity as a robust approach for obtaining rich phenotypic data, and being used in bioprocessing and biofuel development. Mass spectrometry imaging approaches, including several nanotechnologies, provide complimentary information by localizing metabolic processes within complex biological matrices. Together, the two technologies can provide new insights into the metabolism and interactions of cells.

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Introduction

Diverse biotechnologies ranging from synthetic biology to clinical research rely on an accurate understanding of metabolism. Hence, metabolomics methods that characterize and quantify the metabolic processes within the cell have become integral to diverse research avenues. Recently, metabolite imaging methods have been developed to localize metabolism within the biological matrix. Now, exometabolomic methods are emerging to bridge the gap between metabolic profiling and imaging experiments.

Exometabolomics, also known as metabolic footprinting, is the study of how cells transform their small molecule environment. Here metabolomics is used to detect changes in culture media as a result of cell culture, typically, by comparing fresh versus spent media. While simple, this approach provides powerful information on cellular phenotypes while avoiding the challenges of measuring intracellular metabolites. This is because exometabolite levels integrate over minutes or hours versus intracellular metabolites that may be altered or degraded within milliseconds of extraction [1,2] requiring careful quenching of ongoing cellular processes to minimize the effects of cellular stresses encountered in sample preparation (e.g. centrifugation). Allen *et al*. in 2003 showed the exometabolomics approach to be robust to sample preparation methods and could discriminate yeast growth states and mutants using direct infusion electrospray ionization (ESI) mass spectrometry [3••]. More recently exometabolomics has been used as an information-rich method for studying released compounds under varying environmental conditions [4], which will be discussed further throughout this review.

Mass spectrometry is currently the principal method used for exometabolomics [5], though other techniques including NMR are also well suited for these studies [5–7]. While direct infusion ESI enables high throughput screening, liquid chromatography coupled to mass spectrometry (LC–MS) greatly increases metabolite coverage as chromatographic separation limits signal suppression enabling detection of low-abundance or weakly-ionizing metabolites (Figure 1a). A major limitation of LC–MS exometabolomics is that it does not provide information on the localization of these metabolic processes.

Mass spectrometry imaging (MSI) enables direct interrogation of biomolecules within the 3D architecture of cellular environments. This is accomplished by generating gas phase ions from spatially defined locations (e.g. using a laser or focused ESI cone) ultimately to construct a map of ions across a 2D surface. There are a wide-range of MSI approaches, as recently reviewed [8••,9••] including many nanotechnologies such as nanostructure-initiator mass spectrometry (NIMS), Nan post Arrays (NAPA) and many others [10].

Here we review exometabolomics approaches and applications to show how this simple technique can provide critical insights into phenotypes, cellular interactions and environmental transformations. We highlight how this approach can be used in rapid phenotyping assays that are becoming popular in drug development, synthetic

Figure 1

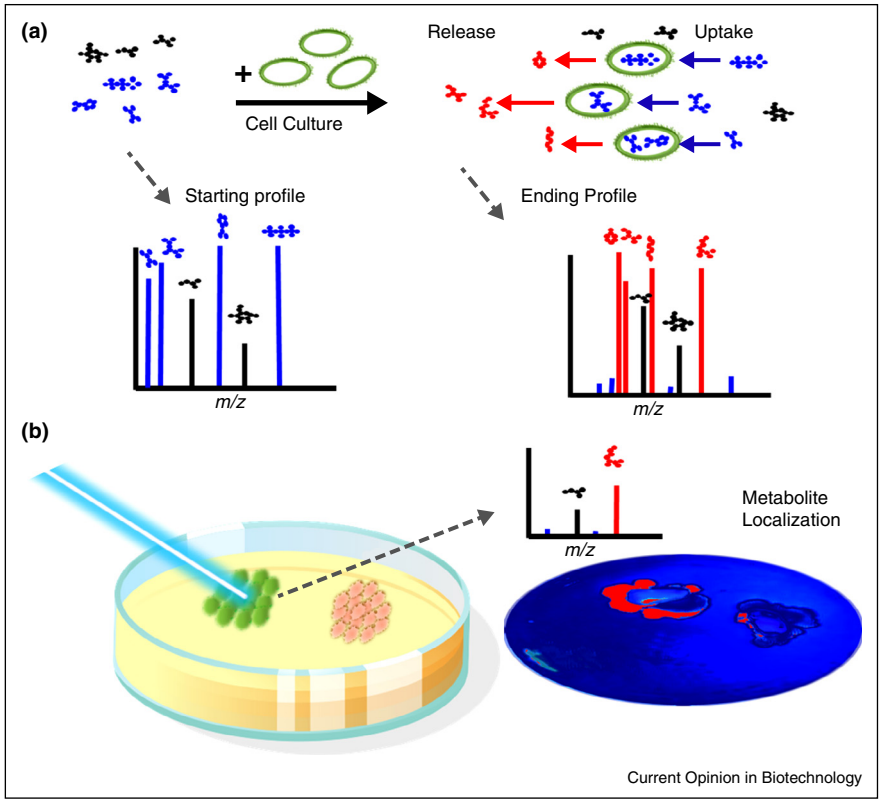


Illustration of exometabolomics workflows. **(a)** In LC–MS based exometabolomics, cells of interest are cultured in media containing metabolites, and these metabolites are measured by LC–MS, before and after culture to define cellular substrates and products. **(b)** In MSI-based exometabolomics, spatially defined desorption/ionization is used to generate images of metabolite composition across cell culture plates.

biology, biofuel development, and investigating the metabolic interactions and dependencies of cells. Lastly, we anticipate that exometabolomics in conjunction with mass spectrometry imaging (MSI) will provide important insights into metabolic interactions between populations of cells.

Phenotypic analysis

The initial report describing metabolic phenotyping focused on differentiating yeast strains using direct infusion. In this study, the media from 19 *Saccharomyces cerevisiae* (yeast) strains with unique deletions were compared, confirming that gene knockouts can be discriminated reproducibly by exometabolomic (AKA footprinting) measurements and statistical analysis [3••]. There are many recent examples of exometabolomic profiling in yeast. This was extended by Chumnanpuen *et al.* to achieve real time sampling of yeast media under three different growth conditions [11] and Castrillo *et al.* used a systems biology approach, including exometabolomics to understand the growth control of yeast for metabolic engineering [12]. Another interesting application is the recent comparison of 69 commercial yeast strains commonly used in chardonnay production. Here, 29 primary and secondary metabolites were measured, and up to 1000-fold differences were noted between strains. The largest variability was noted in the ratios of acetate esters to ethyl esters, which greatly affects the sensory profile (predominantly taste) of wine [13].

We have used exometabolomics to investigate heterotrophy by the cyanobacterium, *Synechococcus* sp. PCC 7002 [14–16]. Here cultures were grown in four different media: minimal media or the same augmented with yeast extract or tissue culture media or extracts of *Synechococcus* sp. PCC 7002. It was found that the cyanobacteria released very few metabolites but took up a wide range of compounds. Interestingly, it appears that the cyanobacteria showed preference for their own metabolites including many novel metabolites.

More recently we examined the use of this approach for high throughput gene annotation using the complete mutant libraries of *Shewanella oneidensis MR1* [17] and the KEIO *Escherichia coli collection* [18]. Here, exometabolomics was used to identify 71 metabolites taken up by the type strains. Ten interesting and commercially

available metabolites were selected for analysis using a 2.2 minute LC/MS method. Over 8000 mutants were individually cultured in media augmented with these 10 metabolites for exometabolomic analysis, where failure to take up a metabolite indicated disruption of an associated gene. This identified genes of known function, novel transporters and enzymes including a histidase for ergothionine [19], a microbial produced metabolite associated with Crohn's disease [20]. Most recently, we have also found that exometabolomics can be used for secondary metabolite analysis from outer membrane vesicles (OMVs), such as in the case of *Myxococcus xanthus*, which secrete small molecules with antibiotic properties into their extracellular environment [21].

Biofuel development

The use of lignocellulosic biomass for biofuel generation has gained popularity in the last decade [22]. Lignocellulosic biomass is predominantly composed of cellulose, hemicellulose and lignin. Lignin is a complex aromatic polymer, which functions as the supportive structure of lignocellulose. Currently, breakdown of lignocellulose requires a pretreatment-hydrolysis step, in order to hydrolyze the rigid polysaccharides into monomers, for microorganisms to utilize the biomass in fermentation processes. This pretreatment, however, releases compounds that can act as inhibitors of the fermentation process, reducing the performance of fermenting yeast [23]. Zha *et al.* used exometabolomics to identify novel toxic inhibitors being released in the pretreatment step to break down lignocellulose [24].

Microorganisms that are capable of mixed-sugar fermentation are also used in the cellulosic biofuel industry, as they have increased inhibitor tolerance. Casey *et al.* used exometabolomics to monitor co-fermentation of glucose and xylose in genetically engineered strains of yeast in the presence of increasing salt concentration. They found that xylose consumption was strongly affected by the presence of salt, and a shift in metabolism to increased glycerol production during xylose fermentation when salt was present. It was concluded that salt concentrations have a negative impact on yeast performance during cellulosic ethanol production [25].

Bioprocesses

Microbes and mammalian cells are often used for industrial production of complex protein therapeutics. Understanding what these specialized cells consume and excrete can aid in designing processes to improve recombinant protein yields, folding properties, and desired therapeutic activity [26]. Carinhas *et al.* looked at the exometabolomic signatures of glutamine synthetase (GS) — Chinese hamster ovary (CHO) clones to understand the variable expression of IgG4, identifying required nutrients to increase IgG generation [6,27]. Carneiro *et al.* used exometabolomics to monitor the metabolic impact of heterologous protein production in *E. coli*. They noticed accumulation of glyoxylate shunt pathway inhibitors in the culture media, and nutritional shifts suggesting routes to replenish TCA cycle intermediates required for the expression of heterologous protein [28].

Other major bioprocessing applications for exometabolomics have focused on increasing yields and for monitoring fermentations. For example Xu *et al.* observed a 27% increase in soluble sugars released from a glycogen synthase null mutant in comparison with wild type *Synechococcus* sp. [29]. Fu *et al.* used exometabolomics to understand the impact of scale-up on the host microorganism's behavior during industrial fermentation [30]. Exometabolomics has also been used in bioprocess development to monitor microbial contamination. Sue *et al.* were able to accurately (97%) classify fermentation samples coming from contaminated cultures, using exometabolomics with GC–MS [31].

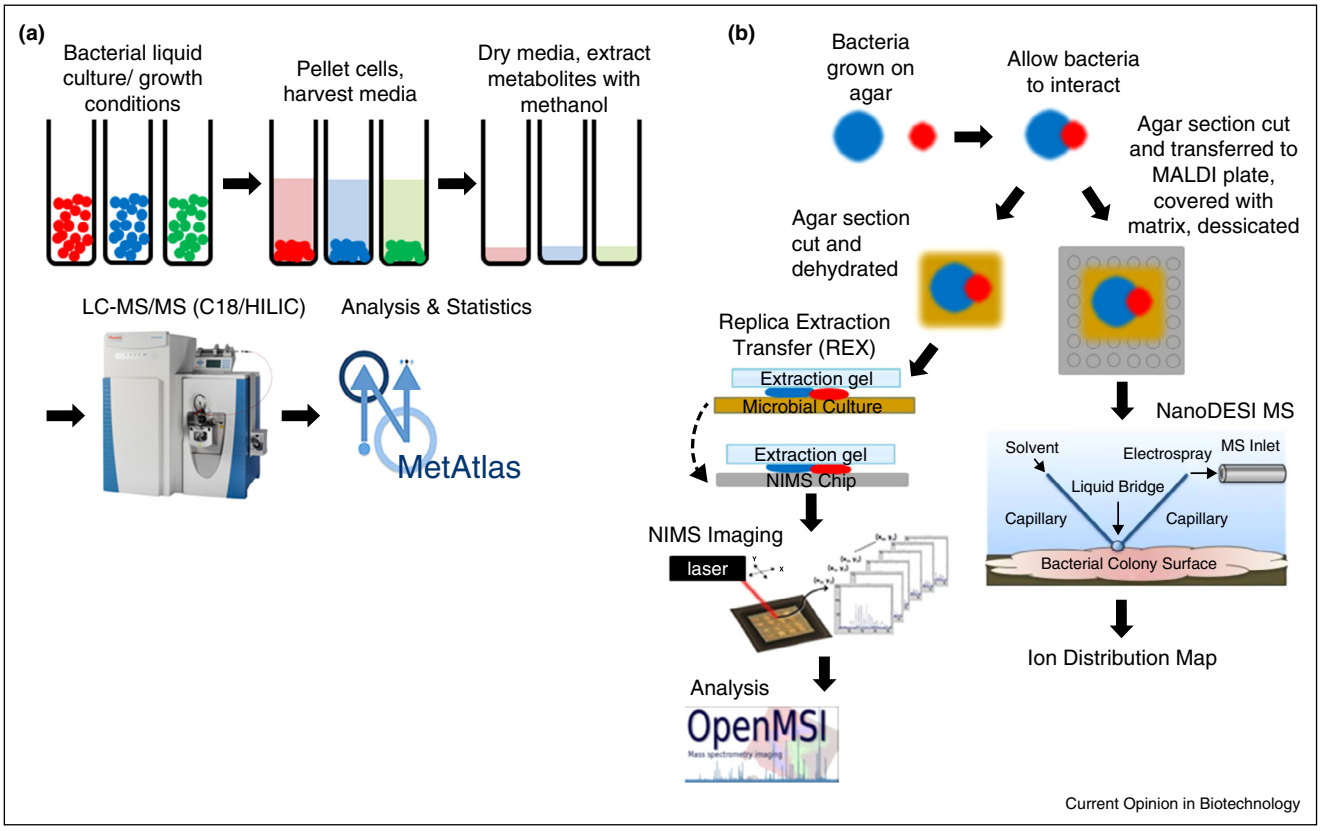
Drug mechanisms of action

A recent development has been to use exometabolomics to characterize the mechanism of action of an ethanolic extract of *Garcinia mangostana* Linn. for the treatment of malaria. Metabolites from the culture medium of the malaria-causing parasite, *Plasmodium falciparum*, were measured after treatment with the extracts. It was found that these extracts resulted in the absence of malate in the culture media which the authors attributed to interruption of TCA cycle metabolism in the parasite [32]. Understanding the mechanism of action of drugs through exometabolomics is a promising research direction, which could potentially be expanded to the investigation of drug toxicity in the future (Figure 2).

Examining cellular interactions

While most articles and reviews published in the field of exometabolomics describe LC–MS-based techniques [38], laboratories have also been using mass spectrometry imaging (MSI) as a complimentary technique that allows for the localization of metabolites in 2D and 3D from tissues [39–42], around biofilms and bacterial colonies [8••,9••,35••,36,43,44•] among many other applications [8••]. MALDI and ESI-based techniques are most widely used for metabolite MSI. While MALDI-MSI has the advantage of being high throughput, ESI-based techniques benefit from a more comprehensive analysis, due to fewer metabolites being detected simultaneously. Additionally, nanometer-scale secondary ion mass spectrometry (nanoSIMS) has been widely used in conjunction with stable isotopes to understand microbial metabolism and the turnover of proteins and metabolites within tissues and cells [45,46]. For example, Mayali *et al.* used nanoSIMS to look at adaptations to nutrient availability in mixed microbial communities within the San Francisco Bay [47]. Similarly, Woebken *et al.* and Fike *et al.* used the

Figure 2



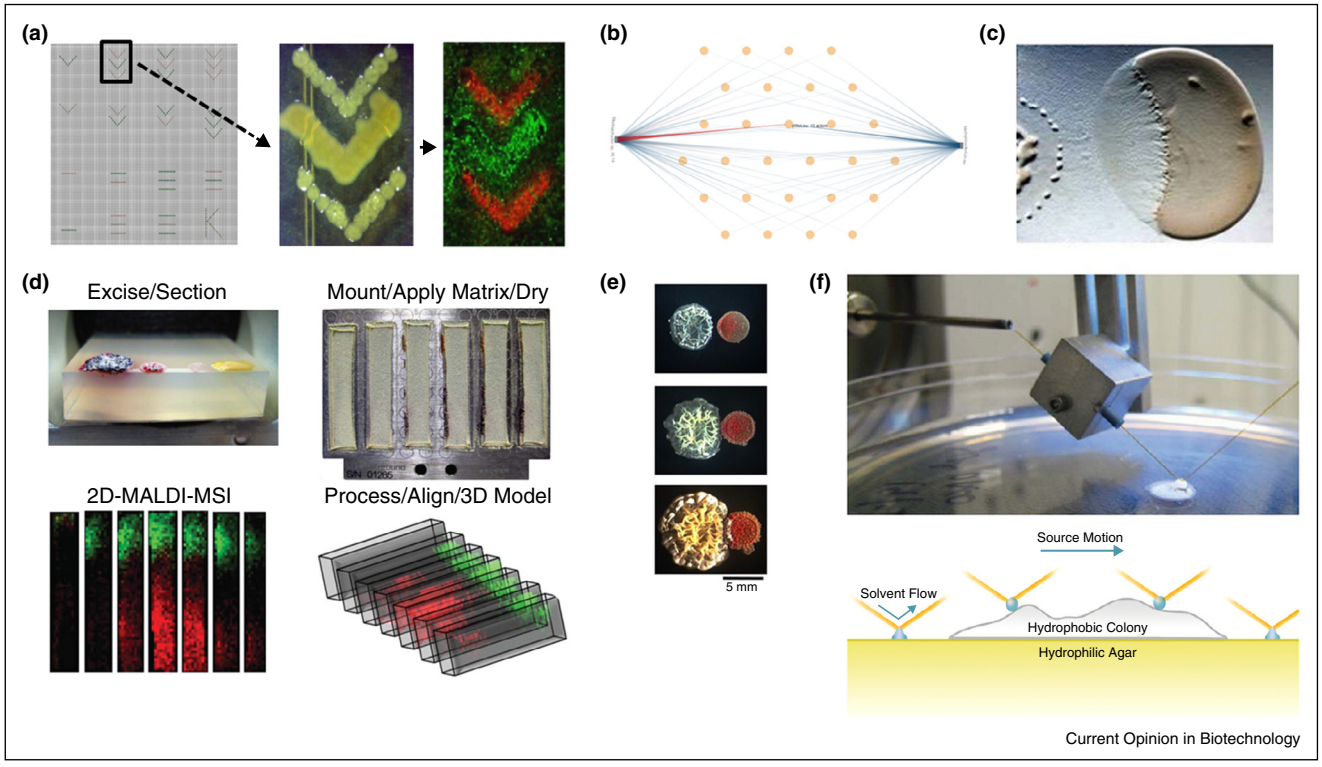
Approaches and technologies used for exometabolomics. **(a)** Protocol for preparation of exometabolites from liquid culture [14,33,34], and **(b)** two protocols for preparation of exometabolites from agar gels [35••,36,37••].

same technology to study N_2 fixation and sulfide levels, respectively, from the hypersaline microbial mats found in Guerrero Negro, Baja California Sur, Mexico [48,49].

The Dorrenstein lab has developed a wide range of approaches for imaging microbial interactions and secondary metabolites. Watrous *et al.* described the use of MALDI imaging to map microbial exchange in three dimensions (3D) [9••]. They cut cross sections of 8 mm deep agar and imaged each one. The images were computationally combined to generate 3D models (see Figure 3d). These models were able to map the chemical distributions of *Candida albicans* in the presence of *Pseudomonas aeruginosa*, and it was discovered that there was increased rhamnolipid production by *P. aeruginosa* [9••]. The Dorrenstein lab has also published on a new concept describing the use of nanospray desorption electrospray ionization (nanoDESI) with MSI to profile metabolites directly from a Petri dish, individually or in a mixed biofilm (Figure 3f) [50]. Additionally, Traxler *et al.* recently used MSI to study the interaction between *Streptomyces coelicolor* with five actinomycetes (Figure 3e). They found that several desferrioxamines were secreted by *S. coelicolor* while interacting with several of the actinomycetes [37••]. Essential data on secondary metabolites, including tandem mass spectrometry data, is available via the Global Natural Products Social Molecular Network (GnPS) (https://gnps.ucsd.edu/ProteoSAFe/libraries.jsp). This database contains secondary metabolite spectra available for public searching from multiple sources.

Nanostructure-initiator mass spectrometry is a highly sensitive surface-based technique for metabolite analysis from tissue sections [39,41,51,52]. However, early efforts to extend this approach to investigate colony interactions were unsuccessful largely due to microbial exopolysaccharides that inhibited desorption/ionization. Recently, the replica-extraction-transfer (REX) technique was developed to allow for the transfer of metabolites directly from agar cultures onto nanostructure initiator mass spectrometry (NIMS) surfaces. These surfaces are advantageous as they allow for the detection of small molecules with very low background [43,51]. Acoustic printing of bacteria allows for the growth of individual isolates in close proximity (Figure 3a), and the sensitivity and spatial resolution of NIMS allows for the detection of metabolites both present on the bacteria, and in the surrounding areas between bacterial isolates. The resulting data is

Figure 3



Examples of mass spectrometry imaging-based exometabolomics. **(a)** Acoustically printed bacteria imaged with NIMS, **(b)** the Web of Microbes exometabolomics data repository for looking at microbial interactions and food webs [53], **(c)** the predatory behavior of M. *xanthus* in the presence of *E. coli* [21], **(d)** three-dimensional MALDI imaging of *P. aeruginosa* and *C. albicans* in co-culture [9••], **(e)** the interaction of *S. coelicolor* with actinomycetes [37••], and **(f)** MSI using nanoDESI directly from agar gels.
The part figure (b) is adapted with permission from Ref. [35••] (Copyright 2014, American Chemical Society). The part figure (f) is adapted with permission from Ref. [50] (Copyright 2013, American Chemical Society).

directly accessible via the browser-based MSI software; OpenMSI [36] (https://openmsi.nersc.gov/openmsi/client/).

Outlook

Exometabolomic studies will provide critical insights into the distributed metabolism occurring in microbial communities and tissues by defining the metabolic inputs and outputs of cell types. Given the importance of cell–cell interactions including signaling and microenvironmental controls, it is important to complement these studies with MSI. Thus this integrated approach takes advantage of the more comprehensive analysis of LC–MS/MS based exometabolomics [8••] and the spatial information provided by MSI. We envision that exometabolomic (e.g. webofmicrobes.com) [53], natural product (e.g. https://gnps.ucsd.edu/ProteoSAFe/libraries.jsp), lipid (e.g. LipidMaps, LipidBlast) [54,55], metabolite (e.g. Metlin, HMDB, MassBank) [56–59] and MSI data repositories (e.g. OpenMSI) will be critical to enable the integration of these diverse data types and improvement of our understanding of cellular metabolic interactions.

A few recent examples illustrate the power of this approach for investigating microbial interactions, where one microbe is releasing a substance that is toxic to a neighboring microbe, such as is the case with the predatory behavior seen by *M. xanthus* in the presence of *E. coli* [21,60] (Figure 3c). Moree *et al.* investigated the interaction between *P. aeruginosa* and *Aspergillus fumigatus*, common opportunistic pathogens in cystic fibrosis, at the molecular level. They were able to visualize the secreted metabolites through MALDI TOF and MALDI FT-ICR based MSI, and it was discovered that secreted phenazines are converted by *A. fumigatus* into other chemical entities with enhanced toxicities [61•].

Conclusion

Exometabolomics provides an experimentally robust approach to obtain rich phenotypic information on cells. It has been used for diverse applications including gene annotation, biofuel development, bioprocessing, and drug mechanisms of action. This approach can be used to define the inputs and outputs of cells to study cellular interactions. While LC–MS is highly sensitive and

provides the largest dynamic range for characterization of large numbers of metabolites, the localization of these metabolites through MSI and targeted MSI provides useful information for understanding how microbes interact in complex environments. The combination of these two complementary approaches, exometabolomics and MSI, will provide critical information on the distributed metabolism occurring in microbial and other cellular communities.

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MALDI-TOF and MALDI-FT-ICR imaging were utilized to study the interaction of *P. aeruginosa* with *A. fumigatus*, commonly found in the mucus of CF patients. It was found that phenazine metabolites secreted by *P. aeruginosa* were converted by *A. fumigatus* into other chemical entities, some with increased toxicity.