Journal of Microbiological Methods 86 (2011) 8–15

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Proton Transfer Reaction Mass Spectrometry detects rapid changes in volatile metabolite emission by *Mycobacterium smegmatis* after the addition of specific antimicrobial agents

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ARTICLE INFO

Article history:
Received 11 November 2010
Received in revised form 17 January 2011
Accepted 21 January 2011
Available online 28 January 2011

Keywords:
Mass spectrometry
PTR-MS
Volatile organic compounds (VOCs)
Drug susceptibility testing
Mycobacteria

ABSTRACT

The metabolic activity of plants, animals or microbes can be monitored by gas headspace analysis. This can be achieved using Proton Transfer Reaction Mass Spectrometry (PTR-MS), a highly sensitive detection method for trace gas analysis. PTR-MS is rapid and can detect metabolic responses on-line as they occur. Here, we study the headspace of actively growing cultures of paired ciprofloxacin sensitive and resistant bacterial strains (*Mycobacterium smegmatis* in Middlebrook M7H9 liquid media) after the addition of the antibiotics ciprofloxacin and gentamicin in real time. Following the emission patterns of the mycobacteria over time allowed volatile markers specific for the bacterial response to each antibiotic to be detected. A proportion of the measured responses were very rapid, occurring within three hours after the addition of the compounds and varied between isolates with different resistance phenotypes. Specifically, we observed a two fold increase of m73 (unidentified C4 compound) within 10 h after the addition of ciprofloxacin and a threefold increase of m45 (acetaldehyde) within 4 h after the addition of gentamicin as compared to values before the addition. Monitoring the emission of specific volatiles into the culture headspace thus has the potential for rapid drug susceptibility testing. Moreover, these and other differences in the measured responses to the two tested compounds provide evidence that monitoring multiple compounds may also give an indication of the mechanism of action of the compound added.

1. Introduction

Rapid determination of the sensitivity to antimicrobial agents is a critical activity in microbiological diagnostics. Methods for automating the bacteriological characterization of clinically isolated bacterial cultures are required. This is particularly true for slow growing organisms such as mycobacteria. Automated culture systems are available for *M. tuberculosis* but culture positive samples must be manually manipulated once grown to confirm the species and determine the susceptibility profile (Hillemann et al., 2005; Somoskovi et al., 2000). Headspace analysis would in principle allow bacterial cultures to be characterized on-line without manual manipulation. It has been shown that it is possible to measure Volatile Organic Compounds (VOCs) from the headspace of bacterial cultures using Proton Transfer Reaction Mass Spectrometry (PTR-MS) (Bunge et al., 2008; Critchley et al., 2004; Kai et al., 2010) or related methods (Allardyce et al., 2006a). Those systems have provided consistent evidence that various microbes and mycobacteria produce distinctive patterns of VOCs. The advantage of PTR-MS is the possibility to rapidly measure on-line the volatile compounds emitted by bacterial cultures without the need for gas sample preconcentration, due to its sensitivity. Thus, monitoring the release of volatile compounds into the headspace of growing cultures in principle provides a method for rapidly measuring bacterial responses to specific compounds. As the release of volatiles is related to bacterial metabolic activity, headspace sampling and analysis in principle allows these responses to be detected in real time before effects on growth rate become apparent. In order to demonstrate the potential of this approach in an organism with metabolic activity comparable to *M. tuberculosis*, we study the response of *M. smegmatis* cultures to the addition of antibiotics in real time. The headspace of various strains of *M. smegmatis* are monitored with and without the addition of ciprofloxacin and gentamicin, two antimicrobial agents with different mechanisms of action, with the aim of detecting VOC emission patterns specific for each phenotype and compound. Once identified and characterized, these responses could be used to rapidly detect drug susceptibility or in principle determine the mechanism of action of drugs.

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doi:10.1016/j.mimet.2011.01.025

In this study we use PTR-MS, which has proven to be a valuable tool for sensitive VOC detection in medical research (Lindinger et al., 1998; Prazeller et al., 1998; Steeghs et al., 2006), atmospheric monitoring (de Gouw et al., 2003; Lindinger et al., 1998), and biological research (Boamfa et al., 2004; Peñuelas et al., 2005; Steeghs et al., 2004). For more analytical power a second mass spectrometer is used in which the quadrupole mass spectrometer is replaced by an ion trap mass spectrometer for MS/MS (Prazeller et al., 2003; Steeghs et al., 2007; Warneke et al., 2004). The first mass spectrometer is used to follow on-line VOC emissions of mycobacteria over a period of days, the second for identification of metabolic gasses.

2. Materials and methods

2.1. PTR-MS

The headspace of *M. smegmatis* bacterial cultures have been analyzed with a home built PTR-MS. The system is a modified version of the PTR-MS described earlier (Boamfa et al., 2004). We will give here only a brief description of the working principles.

In PTR-MS trace gas molecules are ionized via a proton transfer reaction attaching a proton to the trace gas molecules in air, after which they are analyzed with mass spectrometry. In general, the molecules do not dissociate under such a soft-ionization process as is the case with electron impact ionization, which simplifies identification of the gasses and enhances the sensitivity (Lindinger et al., 1998). The PTR-MS consists of (see Fig. 1): an ion source (Fig. 1–1) in which H_3O^+ ions are produced by a discharge in a H_2O–He mixture, a drift tube (Fig. 1–2) in which the proton transfer reaction takes place, a quadrupole mass filter (Fig. 1–3) and a secondary electron multiplier (Fig. 1–4). In PTR-MS trace gasses with a proton affinity higher than that of water are ionized and there is no interference with the main natural constituents in air (N_2, O_2, CO_X, and NO), since their proton affinities are lower than that of H_2O. PTR-MS has the capability to measure simultaneously multiple gasses on-line, rapidly and quantitatively. The mass number of the detected ion is given by the molecular mass of the substance plus the mass of the single proton m_H (mRH^+ in atomic mass units, amu).

For identification of specific masses of interest we also use MS/MS with the help of Proton-Transfer-Reaction Ion Trap Mass Spectrometry (PIT-MS). The characteristics of the PIT-MS are similar to those of the PTR-MS except that an ion trap is used as a mass analyzer (Steeghs et al., 2007) instead of a quadrupole. In the PIT-MS a specific mass can be isolated in the ion trap and forced in collision-induced dissociation (CID) allowing different compounds with identical mass to be differentiated (MS/MS).

Both mass spectrometer systems were calibrated by mixing a variable flow of hydrocarbon-free air, for which a catalytic converter is used at 350 °C, with a fixed flow of 0.3 l/h of a calibrated mixture of N_2 containing 1 ppm (±5%) of ethanol, acetaldehyde, acetone, isoprene, benzene, toluene and xylene (molar masses ranging from 32 to 106 g/mol) (Linde, Dieren, the Netherlands). In this way, a calibration is obtained for these compounds converting ion intensity into normalized counts per seconds (ncps) to gas mixing ratios in part per billion volume (ppbv). From this calibration we calculate the conversion factors for other compounds at other masses (m/z ratios), taking into account the difference in collision rate constant for these gasses in the drift tube and the transmission efficiency factors of the mass spectrometer (de Gouw et al., 2003). Nevertheless, care must be taken when calculating concentrations of masses that have not been identified, as they can be fragments of bigger molecules. The calibration curves of the VOCs present in the calibrated mixture obtained with the PTR-MS are shown in Fig. 2; detection limits vary between 1 and 50 ppbv (signal-to-noise ratio S/N = 2, methanol 1 ppbv, acetaldehyde 14 ppbv, acetone 4 ppbv, isoprene 47 ppbv, benzene 6 ppbv, toluene 8.5 ppbv, xylene 10 ppbv).

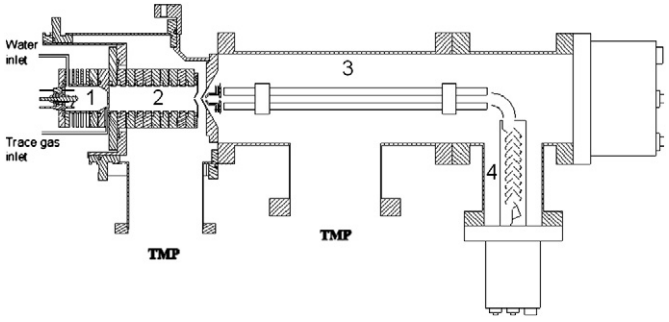

Fig. 1. Schematic diagram of the PTR-MS device. 1) Ion source, 2) drift tube (E/N value 120 Td), 3) quadrupole mass selector, 4) secondary electron multiplier (TMP: turbo molecular pump).

2.2. Bacterial cultures and antibiotic treatment

Two related *Mycobacterium smegmatis* strains were used in this study, which differed in their susceptibility to ciprofloxacin: strain A, sensitive to ciprofloxacin (MIC <7 μg/ml), and strain B, a spontaneous laboratory mutant resistant to >10 μg/ml ciprofloxacin. Liquid cultures in Middlebrook 7H9 medium with oleic–albumin–dextrose–catalase (OADC) (Difco, BD Diagnostics, Sparks, MD) enrichment were prepared by incubating at 37 °C, until an optical density (OD420 nm) of 0.5 to 1 was reached. Then 5 ml of this bacterial culture was inoculated in 75 ml of Middlebrook 7H9 + OADC medium in Erlenmeyer flasks adapted for gas flow sampling. Thus, for headspace measurement, 80 ml of liquid mycobacterial cultures were contained in 250 ml glass flasks with a glass stopper fitted with two Teflon open/close valves acting as inlet and outlet (Fig. 3). Ciprofloxacin (7 μg/ml, antibiotic that acts by inhibiting DNA gyrase (Heifets, 1991)) and gentamicin (55 μg/ml, acts by inhibiting protein synthesis (Heifets, 1991)) were added in 1 ml Middlebrook 7H9 as indicated. In control flasks, 1 ml of Middlebrook 7H9 medium alone was added to control for any effect of adding fresh media on the evolution of volatiles.

2.3. Gas handling set-up

A cotton filter was placed in the inlet and the outlet of the Erlenmeyer flasks to prevent bacterial contamination. The bacteria were kept in an environmental chamber (Sanyo Gallenkamp BV, Breda, the Netherlands) at 37 °C, and the headspace of the cuvettes continuously flushed with 1 l/h dry hydrocarbon-free air controlled by mass flow controllers (Brooks Instrument, Ede, the Netherlands).

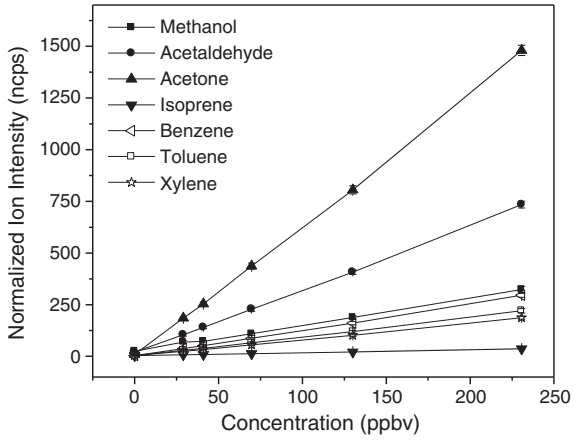

Fig. 2. Calibration curves. Calibration curves for methanol, acetaldehyde, acetone, isoprene, benzene, toluene and xylene showing a highly linear response. When the curves are fitted to a line, the slope of the line represents the amount of normalized counts per second (ncps) per part per billion volume (ppbv).

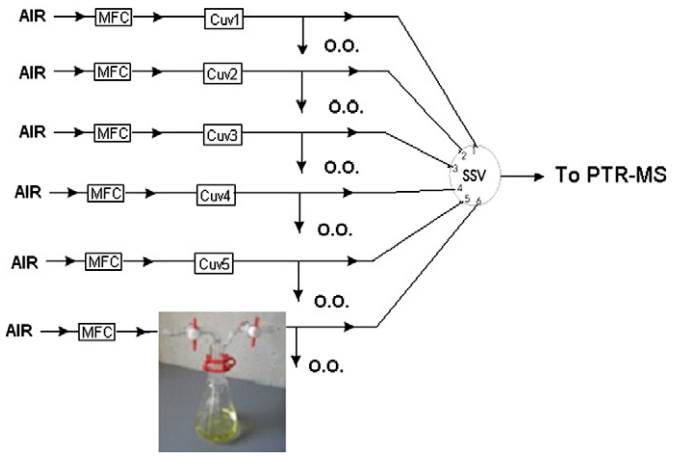

Fig. 3. Gas handling set-up for on-line measurement of bacterial cultures. The insert shows the Erlenmeyer flask. O.O. = overpressure outlets. MFC: Mass flow controllers. SSV: stream selector valve.

Furthermore, the cuvettes were continuously shaken at 70 rpm (Orbital Shaker, Sanyo, Osaka, Japan). All the gas sampling lines and gas connectors were Teflon (Polyfluor Plastics, Oosterhout, the Netherlands). Both sampling lines and drift-tube were heated to 55 °C to reduce memory effects caused by VOC-surface interactions. For each experiment 6 cuvettes were used, of which 4 were filled with bacterial cultures in Middlebrook 7H9 medium and 2 with Middlebrook 7H9 medium alone. The cuvettes were sequentially monitored by switching from cuvette to cuvette using a stream selector valve (Valco cheminert valves, Bester BV, Amstelveen, the Netherlands); each cuvette was sampled for 45 min (Fig. 3). Overflow outlets were placed between the outlet of each cuvette and the stream selector valve to keep a constant airflow through the cuvette while preventing pressure build-up during the period in which the cuvette was not sampled.

2.4. Experimental design

Two types of measurements were performed with PTR-MS for each cuvette. 1) Initially full mass scans were performed on medium alone (negative control) and bacterial cultures over time to identify informative masses. For a full mass scan 5 scans were averaged covering a range from 20 to 150 amu. From this, up to 25 masses were identified as masses at which a high signal could be observed. 2) During the bacterial growth and/or treatment the 25 m/z values were monitored over time for the duration of the experiment, up to a period of six days.

Volatile emissions were followed during the growth of *M. smegmatis* in 4 different situations: (1) Control: no treatment; (2) addition of 1 ml medium only; (3) addition of ciprofloxacin; and (4) addition of gentamicin to which both strains are sensitive. Each experiment was repeated at least 4 times.

3. Results

3.1. Volatile emissions during bacterial growth

Emissions from the flasks containing 75 ml of Middlebrook 7H9 were monitored after the addition of 5 ml of actively growing bacterial cells ($3\times10^8-8\times10^8$ cfu/ml); these emissions were compared with emissions from 80 ml of the culture medium alone. Fig. 4 (panel A) shows the time evolution of the masses with the highest concentrations. Other masses, which showed significant changes in concentration as a result of antibiotic addition (m57, m69, m71, m73, m75, m83, m85, m87, m101), did not show any significant emission change when no antibiotic was added (data not shown). Over the first 24 h of the growth experiment emissions from the bacterial cultures were relatively constant and could not be distinguished from the negative control (medium only). After 24 h a decrease is observed in emissions for specific masses in the inoculated cultures (Fig. 4, panel A). This is most significant for the masses m45 (acetaldehyde) and m47 (ethanol); m27, m29, m39, m41 and m43 showed similar patterns but in lower amounts. M45 (acetaldehyde) and m47 (ethanol) were provisionally identified on the basis of isotopic mass ratios. In this case, the ^{13}C isotopic ratio was used as an indicator of the number of carbons present in a detected compound, considering that ^{13}C constitutes 1.1% of the carbon present. The correlation between the compound detected at m45 (acetaldehyde) and m46 has a value of $(2.55\pm0.01)\%$ (with $R^2=0.997$); the correlation between m47 (ethanol) and m48 has a value of $(2.39\pm0.02)\%$ (with $R^2=0.984$). The predicted isotopic abundances from literature for acetaldehyde and ethanol are 2.3%, which is in reasonable agreement with the experimental values. Therefore, we conclude that the observed species are indeed carbon-2 compounds, supporting our assertion that the two compounds are acetaldehyde and ethanol. The identification of m45 as acetaldehyde was further supported by performing collision induced dissociation (CID) using PIT-MS and comparing its fragmentation pattern with that of pure acetaldehyde (Steeghs et al., 2007).

During the incubation, the acetaldehyde and ethanol concentration levels dropped significantly to below the detection limit of the instrument. After complete depletion of the acetaldehyde and ethanol levels, m59 started increasing, reaching a maximum ~5 h later (Fig. 4, panel A). The identification of m59 (acetone) was based on collision induced dissociation (CID) with the PIT-MS. The use of isotopic ratios helped to determine the number of carbons present in the molecule. From the correlation between m59 and m60, the presence of 3 carbon atoms is inferred, and a compound corresponding with the formula C_3H_6O is expected. However, there are two candidates corresponding to this formula, acetone and propanal. When performing CID on pure acetone, m31 is the most abundant fragment from the two main fragments observed (m41 and m31); on the other hand, pure propanal has the same principle fragments, but has its most abundant fragment at m41 (Warneke et al., 2005). Thus, from the CID patterns performed on the m59, we conclude that this mass is mainly acetone.

By varying the initial inoculum size of *M. smegmatis* and/or the volume of medium (amount of available nutrients), the timing of the depletion of acetaldehyde and ethanol as well as the increase in emission of acetone was altered. This is shown in Fig. 5, where the addition of an identical inoculum of bacteria to a larger volume of medium postpones the decrease in ethanol by approximately 4 h. In the absence of the addition of antibiotics no difference in the emission patterns of the two strains of *M. smegmatis* used was detected (data not shown).

3.2. Volatile emissions after antibiotic treatment

Next, we determined if the addition of an antibiotic to the growing bacterial cultures would result in a measurable and characteristic reaction. Both strains of *M. smegmatis* were investigated, A and B; A is sensitive to ciprofloxacin, while B is a spontaneous, laboratory generated, ciprofloxacin resistant mutant. Both A + B are susceptible to gentamicin (Lab codes A = Msmeg1, B = Msmeg1Res).

In Fig. 6 the effects are shown of adding ciprofloxacin (7 μg/ml) to strain A (1.06×10^8 bacteria/ml) and B ($2.02\times10^8\times$bacteria/ml) and the negative control (medium only) for masses m57, m75, m83 and m101. In this experiment ciprofloxacin was added 29 h after inoculation of the bacterial cultures. For the susceptible strain (A) a clear effect is observed in volatile emission within 6 h. The resistant strain (B) did not show any detectable response after the addition of ciprofloxacin, though a minor increase was observed (Fig. 6)

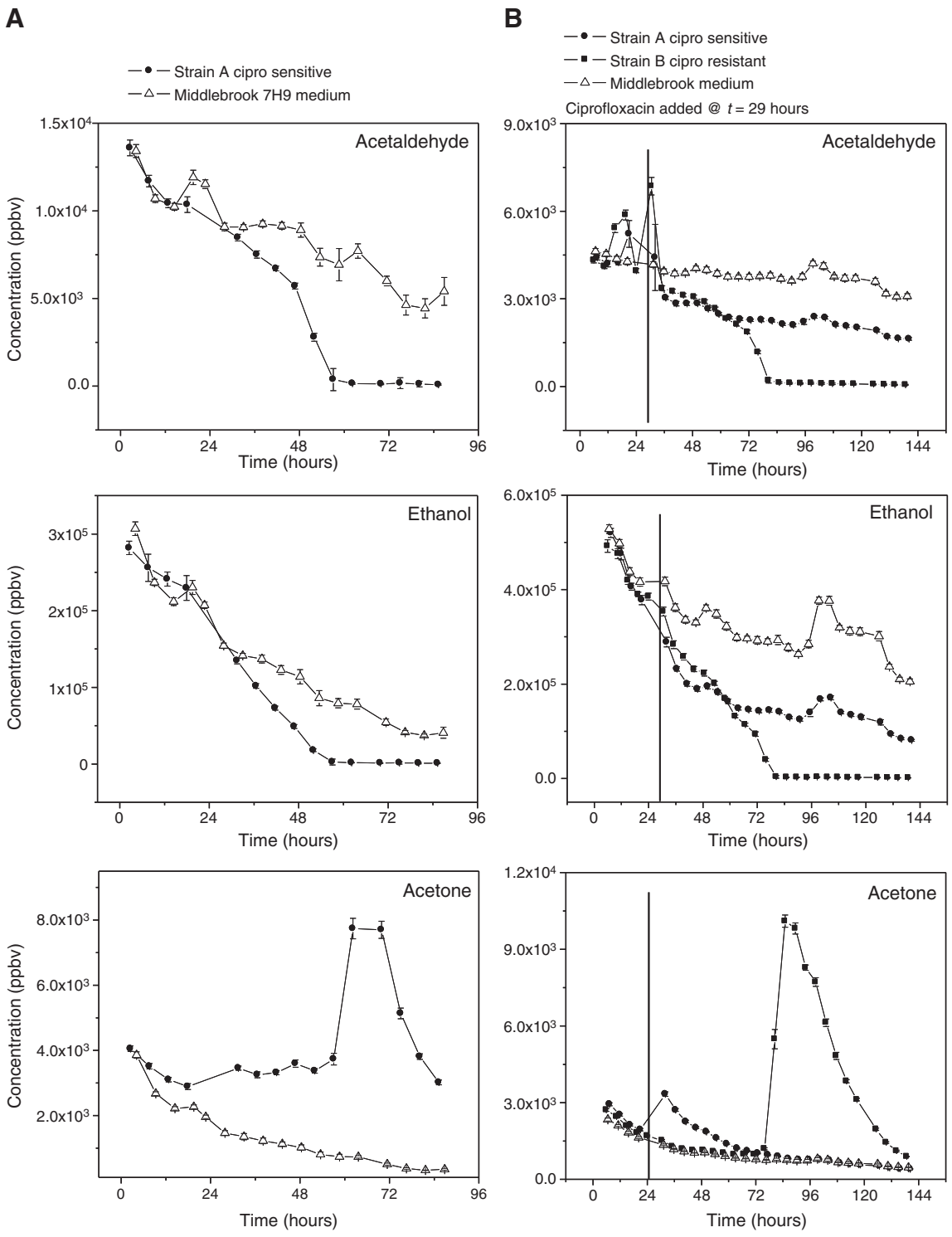

Fig. 4. Acetaldehyde, ethanol and acetone emissions from *M. smegmatis* cultures and medium alone. Panel A: time emissions of acetaldehyde, ethanol and acetone from *M. smegmatis* cultures during growth (filled circles) and medium alone (empty triangles). Panel B: time emissions of acetaldehyde, ethanol and acetone before and after the addition of ciprofloxacin at t = 29 h. The resistant strain B shows the same behavior with and without ciprofloxacin, while the sensitive strain A shows a reduction in the rate of decrease in emissions as compared to the resistant strain B.

immediately before the time point in which acetone levels increased dramatically (Fig. 4B). We believe this minor response is most probably related to a change in metabolism or mild stress associated with the depletion of the media. All specific volatile compounds that are increased in sensitive strain A and did not change significantly in the resistant strain B are detailed in Fig. 8. The control flask, medium alone to which an identical concentration of ciprofloxacin was added at 29 h, did not show any detectable emission change. The acetaldehyde, ethanol and acetone emissions from the ciprofloxacin resistant strain B do not show any significant change after ciprofloxacin treatment. The emission was similar to the control containing the untreated strain A (see Fig. 4, panel A and B, and Fig. 8).

Once a detectable difference in response between resistant strain B and sensitive strain A was confirmed for ciprofloxacin, the response to

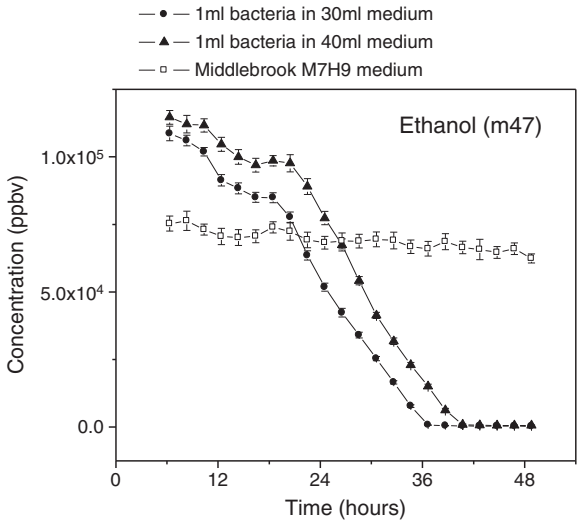

Fig. 5. Influence of the bacterial density on ethanol emissions. An identical inoculum of bacteria (5×10^9 in 1 ml medium) was added to either 30 ml (filled circles) or 40 ml (filled squares) liquid medium at time zero. Empty triangles represent 30 ml of medium alone (negative control). The decrease in ethanol (m47) is delayed when a larger volume of medium is available.

an alternative antibiotic was investigated, in order to discover if the observed VOC emission pattern was a characteristic for the antibiotic, or our method is merely detecting a growth inhibition. For this purpose we selected gentamicin, an antibiotic with a different mechanism of action, to which both strains A and B are sensitive.

In the experiment shown in Fig. 7 gentamicin (55 μg/ml) was added 27 h after inoculation to *M. smegmatis* bacterial cultures (initial concentration = 1.3×10^8 bacteria/ml) and medium alone. As can be seen in Fig. 7, the emission of m45 (acetaldehyde), m57, m73 and m87 have already detectably increased at the first sampling after the addition of gentamicin (three hours), while no significant increase is observed when gentamicin is added to the media alone. An increase in 5 additional masses, although in some cases not so dramatic, was also detected after the addition of gentamicin (see Fig. 8).

In Fig. 8 a summary of the volatiles that detectably increased after the addition of the antibiotics to both strains is presented, next to acetaldehyde, ethanol and acetone. The values express the ratio between the emissions 12, 24 and 48 h after the addition of the antibiotic divided by the values immediately before the addition. Strain A is sensitive to both antibiotics and indeed there are common masses that are emitted for both antibiotics. In contrast, some masses are specific for only one antibiotic, for example m101 for ciprofloxacin and m45 for gentamicin. Both strains A and B are equally susceptible to gentamicin and show indistinguishable emission profiles, and thus the data from both strains was combined for gentamicin.

4. Discussion

In this study we demonstrate that monitoring acetaldehyde, ethanol and acetone in the headspace of *M. smegmatis* cultures provides information about bacterial metabolic activity which can be

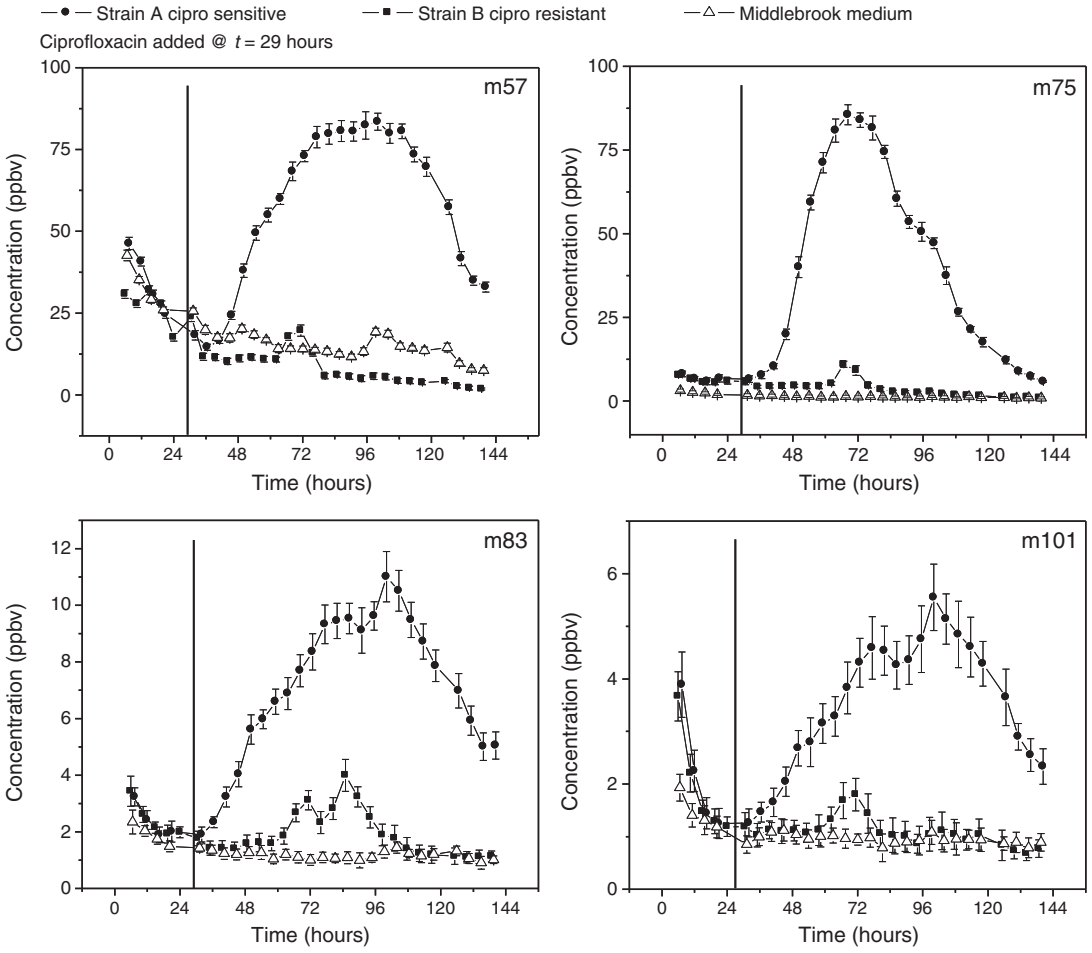

Fig. 6. Effect of ciprofloxacin on *M. smegmatis* headspace. Time profile of VOC emissions for the most significant masses m57, m75, m83 and m101 after the addition of ciprofloxacin (7 μg/ml) at t = 29 h (black bar) to strain A (ciprofloxacin sensitive, filled squares), strain B (ciprofloxacin resistant, filled stars and Middlebrook 7H9 medium alone, empty triangles). For the cultures 5 ml of bacterial with a density of 1.7×10^8 bacteria/ml were inoculated in a 75 ml of media at t = 0 h.

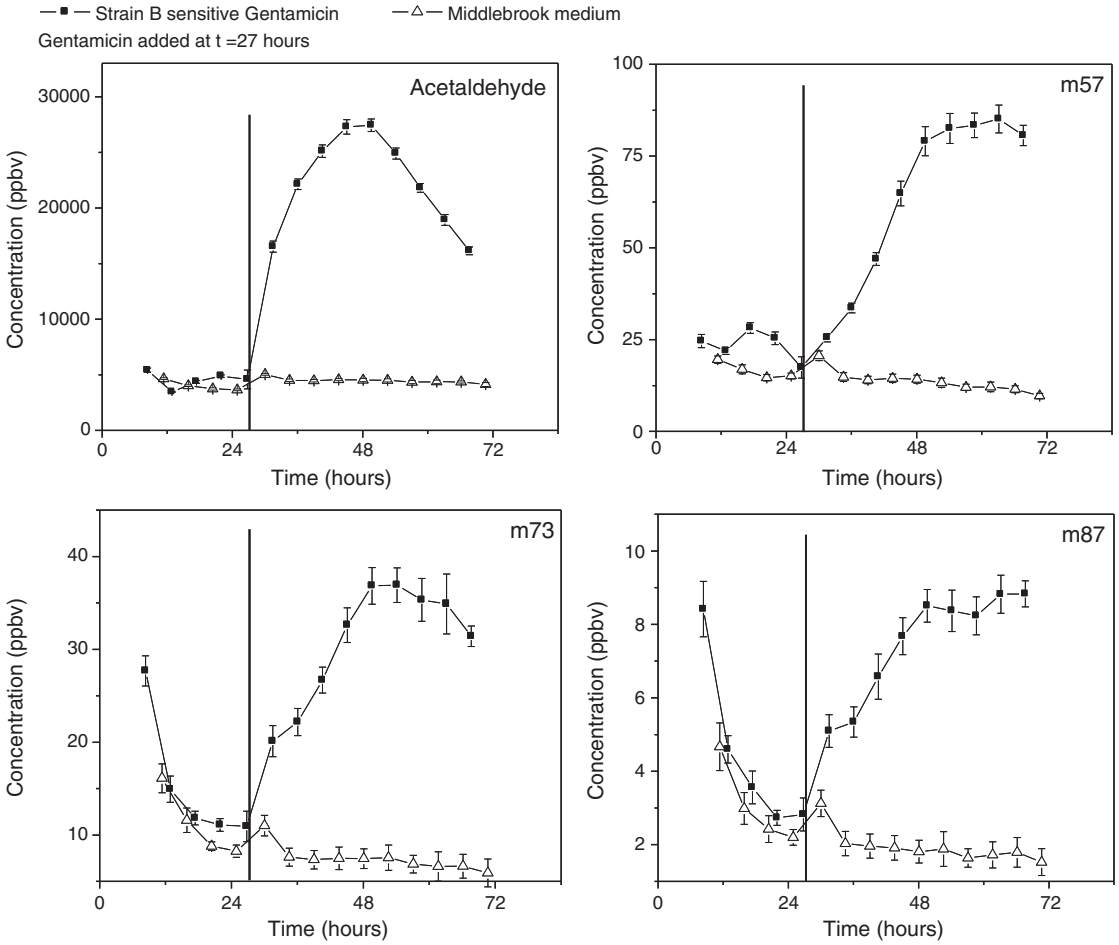

Fig. 7. Effect of gentamicin on *M. smegmatis* headspace. Time profile of VOC emissions of acetaldehyde, m57, m73 and m87 after the addition of the antibiotic gentamicin (55 μg/ml) at t = 27 h (black line) to *M. smegmatis* culture (filled squares) and Middlebrook M7H9 media (open triangles). For the bacterial cultures 5 ml of bacterial culture with a concentration of 4.3×10^8 bacteria/ml were inoculated in 75 ml of medium at t = 0.

linked to the growth phase. The observed degradation of ethanol and acetaldehyde during bacterial growth has been previously reported for bacteria (de Carvalho and da Fonseca, 2005; Osborne et al., 2006), while acetone is a known fermentation product from other bacterial species (Prescott et al., 2005). We see that when the levels of acetaldehyde and ethanol become undetectable there is immediately an increase in acetone levels, which suggests a change in metabolism as was also detected for a mixed microbial consortium (Bustard et al., 2000). There are at least two possible pathways that could be responsible for this switch to acetone production, the one used by *Clostridium acetobutylicum* (Prescott et al., 2005) and the one suggested for propane-oxidizing bacteria (Steffan et al., 1997). Although, it is beyond the scope of this study to determine if or which of these pathways was followed, mycobacteria are also known to undergo a metabolic switch in response to nutrient starvation (Garton et al., 2008).

If we consider the growth curve of *M. smegmatis*, the initial lag phase coincides with the period in which emissions of acetaldehyde and ethanol are higher for the bacterial cultures than for the negative control (medium only) (Fig. 4, panel A). The decrease in bacterial emissions of acetaldehyde and ethanol to levels below that produced by the medium alone appears to be associated with the entry into the log phase, in which the bacteria divide rapidly and exponential growth takes place. Finally, we believe that the subsequent dramatic change from acetaldehyde/ethanol consumption to acetone production is a result of the switch from exponential to stationary phase, when the medium can no longer support exponential growth; thus, the sudden increase in acetone levels appears to be a marker for the entry into the stationary phase, as previously reported for a mixed microbial consortium by Bustard et al. (2000).

Metabolic disruption also occurs when bacteria are exposed to toxic compounds. Indeed the use of headspace analysis has previously been proposed as a method to rapidly detect the response of a bacterial culture to antibiotics (Allardyce et al., 2006b) before the effect on growth would become apparent. Rapid susceptibility testing of pathogens such as *M. tuberculosis* is a subject of considerable interest (Young et al., 2008). *Mycobacterium smegmatis* is related to *M. tuberculosis*, but grows more rapidly and does not require such stringent bio-safety precautions so it is a useful model system for our proof of concept study. Our ability to monitor the headspace of multiple cultures on-line provided an opportunity to further study these responses to two anti-microbial compounds in *M. smegmatis*.

Thus, in addition to the VOC emission profiles dependence on the growth phase we also observed two distinct effects when an antibiotic was applied to sensitive cultures. Firstly, we identified that some volatiles, which showed dramatic changes during normal growth stabilize, and return to levels shown by the medium alone. Secondly, some characteristic responses were also detected, a number of which were common to both antibiotics tested, whereas others were specific for one antibiotic. Most importantly, these responses were detectable very quickly (quicker than the depletion of the medium), some being evident on the first sampling performed only a few hours after the addition of the antibiotics. Identification of these volatile compounds was not possible. The use of PIT-MS did not provide extra information about the identity of the volatiles in this case, due to the low concentration of the characteristic responses appearing after the

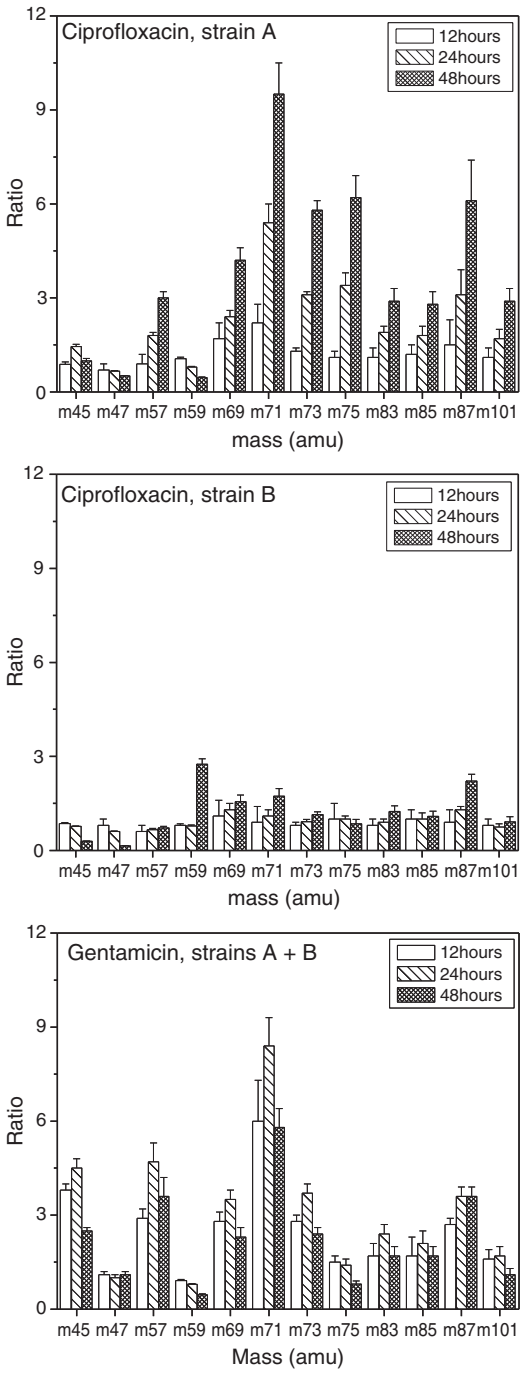

Fig. 8. Evolution over time of masses of interest after the addition of antibiotics. The bars represent the ratio between the concentrations 12 (empty bars), 24 (sparse patterned bars) and 48 (dense patterned bars) hours after adding antibiotics and the concentration immediately prior to the addition of the antibiotics. The values are an average of 4 experiments. Strain A ciprofloxacin sensitive, strain B ciprofloxacin resistant, A + B gentamicin sensitive. Error bars indicate +1 SD.

addition of antibiotics, and the lower sensitivity of the PIT-MS as compared to the quadrupole based PTR-MS. For this purpose, a complementary spectrometric technique such as GC-MS, that allows sample pre-concentration would be more suitable.

In conclusion, we have been able to detect differences between sensitive and resistant strains of *M. smegmatis* by measuring rapid changes in their volatile emission profiles in response to the addition of antibiotics. We believe these responses warrant further investigation; for example, a library could be built with the volatile response of different bacteria to different antibiotics or antibiotic classes. Potentially, this would allow the volatile response to be used to rapidly determine an organism's susceptibility to an antimicrobial compound and possibly even determine the mechanisms of resistance. This method is particularly appealing as manual manipulation of cultures is not required, merely headspace sampling.

Moreover, if certain chemical species can be specifically linked with a particular sensitivity profile, as appears to be the case in this limited proof of concept study, these compounds could be targeted for rapid sensitive detection of bacterial culture response to antimicrobials using simple/inexpensive detectors. Such detection methods are an expanding field of research (Fend et al., 2006; Bruins et al., 2009), for example the development of polymers which undergo a color change in response to the presence of specific volatiles (Rakow and Suslick, 2000), or gold-nanoparticles based cross-reactive chemiresistors that respond to a specific mixture of VOCs (Peng et al., 2009).

Acknowledgements

This work was partly supported by FIND (Foundation for Innovative New Diagnostics).

The authors would like to thank Cor Sikkens for his technical assistance.

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