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Early release of 1-pyrroline by *Pseudomonas aeruginosa* cultures discovered using ambient corona discharge ionization mass spectrometry†

Longhua Hu,[a] Juchao Liang,[b] Konstantin Chingin,*[b] Yaping Hang,[a] Xiaoping Wu[c] and Huanwen Chen*[b]

Pseudomonas aeruginosa (PA) is a leading cause of nosocomial infections in humans with an increasing number of health-threatening implications, which urges faster clinical detection of this pathogen. In the present study, we discovered for the first time the early release of 1-pyrroline vapor by PA cultures using direct ambient mass spectrometry (AMS) analysis of the bacterial culture headspace based on corona discharge ionization. Importantly, the concentration of 1-pyrroline in PA cultures was found to greatly increase during the lag phase and early log phase of bacterial growth (3–6 h, 200–800 ppb), enabling early detection. Typically, 1-pyrroline produced by PA cultures could already be detected in our experiments after 0.5–4 h of incubation at the clinically relevant initial bacterial counts. A reference AMS screen of common infectious microbes from other genera, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Klebsiella oxytoca*, *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Enterococcus faecalis*, *Enterobacter cloacae*, did not reveal a notable release of 1-pyrroline. Our results indicate the high suitability of volatile 1-pyrroline for the early and reliable diagnosis of *Pseudomonas* infections using commonly available MS instrumentation.

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1. Introduction

Pseudomonas aeruginosa (PA) is an opportunistic, nosocomial pathogen which frequently causes pneumonia, urinary tract infections, bacteremia, surgical wound infections, osteomyelitis, and various other diseases.[1] PA exhibits multi-resistance to commonly used antimicrobials, and therefore the effective eradication of PA infections requires early identification and targeted medical treatment.[2] Clinical diagnosis of a PA infection usually relies on microorganism identification in a clinical laboratory, which is laborious, time-consuming (2–3 days) and carries a risk of misidentification.[3] Hence there is increasing attention being paid to analytical tools with higher molecular specificity and speed of analysis.[3–5]

Among a variety of strategies, identification of bacteria *via* the direct analysis of emitted volatile organic compounds (VOCs) is particularly attractive, owing to the non-invasiveness, practical simplicity, cost-efficiency, and toxicological safety.[6,7] A number of workflows have been developed for the rapid identification of bacteria, mostly based on VOC analysis using chemical sensing or mass spectrometry detection.[8–11] Although the reported methods have many evident analytical merits, their integration into clinical practice is hindered due to the requirement of dedicated instrumentation. For example, the identification of most specific PA volatiles such as hydrogen cyanide[4] and methyl thiocyanate[12] requires selected ion flow tube mass spectrometry (SIFT-MS),[13] which is yet not widely available in analytical laboratories. Other VOCs identified in PA cultures have lower pathogen specificity and typically occur at trace amounts, which necessitate sample collection.[14,15] Currently, there is no reliable biomarker for the early recognition of PA using commonly available analytical instrumentation.

In ambient mass spectrometry (AMS), ions are formed outside the mass spectrometer without sample preparation or separation.[16–18] AMS can be implemented on any type of a mass spectrometer with atmospheric interface (*e.g.*, linear ion trap, time-of-flight, triple-quadrupole, Orbitrap) commonly available in bioanalytical laboratories and core facilities. A large variety of AMS methods for the direct molecular analysis of complex samples have been developed over past decade, including desorption electrospray ionization (DESI-MS),[19] direct analysis in real time (DART-MS),[20] laser ablation electrospray ionization

[a]*The Second Affiliated Hospital of Nanchang University, Nanchang 330006, China*
[b]*Jiangxi Key Laboratory for Mass Spectrometry and Instrumentation, East China University of Technology, Room 804, Sci. & Tech. Building, 418 Guanglan Road, Nanchang 330013, Jiangxi Province, P. R. China. E-mail: chingin.k@gmail.com; chw8868@gmail.com; Fax: +86 791 83896370; Tel: +86 791 83896370*
[c]*Department of Infections, The First Affiliated Hospital of Nanchang University, Nanchang 330006, P. R. China*

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(LAESI-MS),[21] low-temperature plasma probe (LTP-MS),[22] paper spray ionization,[23,24] leaf spray ionization,[25] rapid evaporative ionization (REIMS),[26] desorption atmospheric pressure chemical ionization (DAPCI-MS)[27] and many others. Dedicated AMS approaches for bacterial differentiation have been reported based on the ambient desorption/ionization of nonvolatile metabolites (most commonly, lipids) from intact bacterial cells.[28–34] Further, differentiation of bacteria has also been demonstrated based on the AMS of released VOCs using ambient ionization by electrospray droplets[35,36] and atmospheric corona discharge.[37,38] Compared to SIFT-MS, ionization of volatile molecules in AMS is usually less selective, but the possibility of tandem mass analysis in AMS greatly facilitates identification of signals.

In present study, the VOCs emitted by common infectious microbes were analyzed using tandem AMS analysis of bacterial culture headspace based on corona discharge ionization. The method is a variation of the classical atmospheric pressure chemical ionization (APCI)[39] in which bacterial VOCs are transported to the tip of the discharge needle using room-temperature nitrogen gas without carrier solvent and accessory heating. Using this simple approach for the direct VOC analysis, we discovered the release of 1-pyrroline produced by PA cultures during the lag and early log growth stage, after 0.5–4 h incubation. 1-Pyrroline signal could be detected in PA cultures at the initial counts <100 colony-forming units (CFU) per mL, which is far below clinically relevant concentrations. Reference AMS screen of common infectious microbes from other genera did not reveal notable release of 1-pyrroline. Our results strongly indicate the excellent potential of targeted 1-pyrroline detection for the much earlier recognition of PA compared to common clinical diagnostic routines (2–4 h *vs.* 2–3 days). Beneficially, 1-pyrroline detection is achieved using widely available type of MS instrumentation with high simplicity, speed and cost efficiency of operation.

2. Materials and methods

2.1. Bacterial culture

PA isolates were incubated in 10 mL centrifuge tubes (Solarbio, Beijing, China) containing 5 mL aqueous medium at 35 °C and 150 rpm. Two types of liquid media were used: tryptic soy broth (20 g L^{-1} tryptone and 5 g L^{-1} NaCl) and Luria-Bertani broth. PA cultures were grown at four different original PA concentrations (5×10^2 CFU mL^{-1}, 5×10^3 CFU mL^{-1}, 5×10^4 CFU mL^{-1} and 3×10^6 CFU mL^{-1}) and at different incubation times (10–15 time points within total 25 h). For each incubation time and initial PA concentration, at least four replicate samples were independently prepared and analyzed. Growth media without bacteria were cultured under identical conditions for reference analysis.

Sputum and urine from seven healthy volunteers were divided into four equal batches. Simulated sputum cultures were prepared at the initial PA concentration 2×10^5 CFU mL^{-1} by mixing 1 mL of sputum inoculated by 1×10^6 CFU mL^{-1} PA with 4 mL medium solution. Simulated urine cultures were prepared at the initial PA concentration 2×10^4 CFU mL^{-1} by mixing 1 mL of urine inoculated by 1×10^5 CFU mL^{-1} PA with 4 mL medium solution. The four batches from each volunteer were incubated for four different time periods (0 h, 2 h, 4 h, 6 h). In total, 28 sputum and 28 urine cultures were analyzed. Informed consent was obtained for any experimentation with urine and sputum samples.

2.2. AMS analysis of bacterial VOCs

AMS analysis of bacterial VOCs directly from the headspace of incubated tubes was done on commercial ion trap mass spectrometers (LTQ-XL and Orbitrap-XL, Thermo Scientific, San Jose, CA, USA) interfaced with a home-made corona discharge ionization source as detailed in our earlier studies.[37,38] Briefly, +4 kV was applied to a stainless steel needle (OD 150 μm) with a sharp tip (curvature radius ~ 7.5 μm) to create corona discharge at ambient pressure. The headspace VOCs of bacterial cultures were continuously transferred into ionization region *via* plastic tubing (ID 1.0 mm) assisted by nitrogen gas flow (0.1 MPa, 1 L min^{-1}). The angle between the discharge needle and the sample tubing was 30°. The distance from the tip of the needle to the end of the outlet tubing was 2 mm. The distance from the tip of the needle to the inlet of the LTQ capillary was 6 mm. Mass spectra for each sample were accumulated for at least 10 s. The spectra of pure culturing medium incubated over the same time period without PA were collected as background spectra. Reference standard compounds were analyzed from aqueous solutions under identical experimental conditions. Collision-induced dissociation (CID) analysis of 1-pyrroline and reference authentic isomeric compounds was done on LTQ-XL mass spectrometer (Thermo Scientific, San Jose, CA, USA).

2.3. LC-MS analysis of bacterial VOCs

PA cells were filtered out by centrifugation, and supernatant solution was heated to 90 °C. VOCs were collected under ambient conditions for 10 min onto the bottom surface of a glass slide placed 1 cm above the solution. The glass slide was passively cooled by dry ice powder placed on its top surface. The collected condensate was scraped from the slide into an Eppendorf tube using a clean spatula, melted at room temperature, centrifuged to remove possible contaminant particles and then directly analyzed by LC-LTQ-MS (Thermo Scientific, San Jose, CA, USA; C18: length 150 mm; bead diameter 4.6 mm). LC solvent (water/methanol: 20%/80%) was run without gradient at a flow rate of 200 μL min^{-1}. VOCs of reference standard compounds dissolved in water were collected and analyzed under identical experimental conditions.

2.4. Chemicals

Authentic 3-pyrroline (95% purity), *n*-butyronitrile (99% purity), isobutyronitrile (99% purity), *n*-propyl isocyanide and isopropyl isocyanide (97% purity) for reference experiments were purchased from Sigma-Aldrich. *N*-Propyl isocyanide (99% purity) was purchased from AR, J&K Bailingwei (Beijing, China). 1-Pyrroline is not commercially available due to its poor chemical stability. In present study 1-pyrroline was obtained at 54% purity as a kind gift from Prof. Gao Chen and Prof.

Chengfeng Xia (Kunming Institute of Botany). 1-Pyrroline synthesis has been described in a recent publication.[40] Milli-Q water (18.2 MΩ cm) was prepared in house. Methanol was purchased at HPLC purity from TEDIA Co Inc., (Fairfield, Ohiao, USA).

3. Results and discussion

3.1. High-resolution AMS analysis of PA volatiles

Fig. 1 shows a typical mass spectrum of volatiles emitted by PA cultured in 5 mL aqueous medium (20 g L^{-1} tryptone and 5 g L^{-1} NaCl) for 1.5 h recorded using commonly available linear ion trap MS instrument (LTQ, Thermo Scientific, San Jose, CA, USA). The spectrum was dominated by an unknown signal at *m*/*z* 70. Based on high-resolution mass measurements (Orbitrap XL, Thermo Scientific, San Jose, CA, USA), the exact *m*/*z* value of this signal was determined as *m*/*z* 70.066. $C_4H_8N^+$ is the only possible chemical formula that fits this value within the mass accuracy of Orbitrap detection ($\Delta \approx 10$ ppm). All other possible chemical formulas with the nominal mass 70 differ from *m*/*z* 70.066 by >150 ppm.

3.2. CID analysis

The structural CID analysis of $C_4H_8N^+$ ion displayed the major characteristic fragments at *m*/*z* 43 and *m*/*z* 28 (Fig. 2a). Using the same experimental conditions, we performed reference analysis of model volatile metabolites discovered in bacteria, humans and other living organisms that could form $C_3H_8CN^+$ ions upon protonation.[7,41] Out of the studied reference compounds, only protonated 1-pyrroline cations and 3-pyrroline cations produced CID pattern with exactly the same intensity ratios for product ions as in the CID spectrum of *m*/*z* 70 signal from PA culture (Fig. 2a–c). The protonated cations of other model compounds, including *n*-butyronitrile, isobutyronitrile, *n*-propyl isocyanide and isopropyl isocyanide, were also found to yield the CID fragments at *m*/*z* 28 and *m*/*z* 43 but with notably and reproducibly different intensity ratios (Fig. 2d–g). This data strongly suggest that the $C_3H_8CN^+$ ion detected in the volatile headspace of PA cultures was formed by the protonation of pyrroline.

3.3. LC-MS analysis

To further validate the structural assignment of $C_3H_8CN^+$ ion to protonated pyrroline cation as well as to identify the specific pyrroline isomer produced by PA cultures, we also performed LC-ESI-MS analysis of VOCs from the headspace of PA culture as well as LC-ESI-MS analysis of VOCs from the headspace of reference 1-pyrroline and 3-pyrroline aqueous solutions. Note that the observation of pyrroline signal by LC-ESI-MS directly from the supernatant solution of a PA culture was suppressed due to a high salt content in the culturing medium. Nonvolatile salt interferences were efficiently removed by collecting VOCs on a cold glass surface and then analyzing the condensate as described in Experimental section. Fig. 3 shows single ion chromatograms for the *m*/*z* 70 signal, which clearly indicate the selective generation of 1-pyrroline isomer by PA cultures. The chromatographic separation of 1-pyrroline and 3-pyrroline was easily achieved due to the notable difference of their pK_a values ($\approx$6.7 for 1-pyrroline; $\approx$10.5 for 3-pyrrolnie).[42] To our knowledge this is the first evidence for the release of 1-pyrroline by PA.

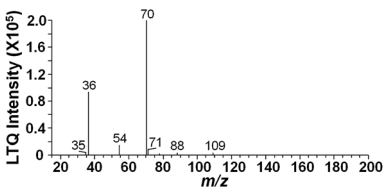

Fig. 1 AMS spectrum of volatiles emitted by a PA culture after 1.5 h incubation, featuring an abundant signal at *m*/*z* 70.

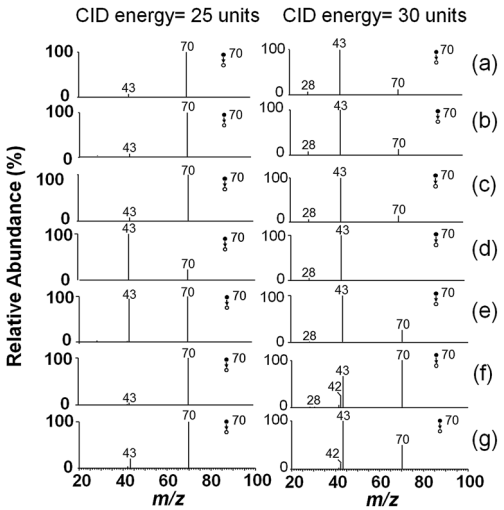

Fig. 2 Collision-induced dissociation of VOC signal at *m*/*z* 70 produced by AMS from the headspace of (a) PA culture; (b) 1-pyrroline; (c) 3-pyrroline; (d) *n*-butyronitrile; (e) isobutyronitrile; (f) *n*-propyl isocyanide; (g) isopropyl isocyanide. CID was performed at the collision energy of 25 instrument units (left column) and 30 instrument units (right column).

3.4. Time profile of 1-pyrroline release by PA

We found that the release of 1-pyrroline by PA cultures followed highly characteristic transient time profile. Fig. 4 shows the time profiles of 1-pyrroline signal from the headspace of cultures with

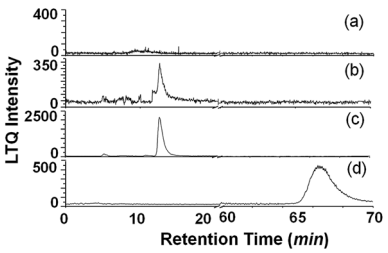

Fig. 3 Single ion chromatogram of *m*/*z* 70 signal in LC-ESI-MS of vapor condensate collected above the aqueous solution containing (a) pure culture medium; (b) PA culture (after 12 h incubation); (c) 1-pyrroline in pure culture medium; (d) 3-pyrroline in pure culture medium. The CID pattern of *m*/*z* 70 signal produced by LC-ESI-MS fully matched the CID pattern of *m*/*z* 70 signal produced by AMS.

different initial counts of PA (5×10^2 to 3×10^6 CFU mL^{-1}). Each time point for different initial PA concentrations was analyzed by AMS using at least four independently grown cultures. Standard deviation of signal response was mostly in the range of 3–8%. In total, *ca.* 400 PA cultures were analyzed (four different original PA concentrations in TSB medium; two different original PA concentrations in LB medium × 10–15 time points × 4–5 replicate samples + 28 urine cultures + 28 sputum cultures ≈ 400 cultures). In all the cultures, 1-pyrroline production was substantially enhanced during the lag phase and early log phase of bacterial growth (3–6 h, Fig. S-1†). We also observed that the higher initial counts of PA resulted in the earlier onset of 1-pyrroline release (Fig. 4). For example, at the initial PA count of 3×10^6 CFU mL^{-1}, notable release of 1-pyrroline was observed after 30 min of incubation, early in the lag phase preceding bacterial growth (Fig. S-1†). The initial increase of 1-pyrroline level in PA cultures was followed by a steady decrease lasting for >20 h. The enhancement of 1-pyrroline emission occurring in the early phase of bacterial culture is particularly beneficial for the rapid identification of PA.

3.5. Quantification of 1-pyrroline release by PA

Quantification of 1-pyrroline in PA cultures was done based on the comparison of 1-pyrroline signal intensity with reference AMS measurements of standard 1-pyrroline dilutions in pure culture medium (Fig. S-2†). The data indicated that the peak solution concentration of 1-pyrroline in PA cultures was in the range from 200 ppb to 800 ppb depending on the original number of PA cells (Fig. 4). The quantitative evaluation of 1-pyrroline concentration derived from AMS analysis was consistent with more rough estimations based on analogous LC-MS analysis. The limit of detection for 1-pyrroline in aqueous solution using AMS analysis of vapor phase was *ca.* 10 ppb.

3.6. Specificity of pyrroline release

Besides PA, the early release of 1-pyrroline was also discovered in two taxonomically close bacterial strains, *P. putida* and *Burkholderia cepacia* (Fig. S-3†). In fact, *B. cepacia* was originally classified as *Pseudomonas* and has only recently been reclassified to *Burkholderia*. The time profile of 1-pyrroline release was very similar in different culturing media (tryptic soy broth, Luria-Bertani broth). Importantly, the reference AMS screen of common infectious microbes, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Klebsiella oxytoca*, *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Enterococcus faecalis*, *Enterobacter cloacae*, did not reveal notable release of 1-pyrroline (Fig. S-3†). These results indicated the high specificity of 1-pyrroline to *Pseudomonas* and to PA, which is the most common *Pseudomonas* infection in humans.

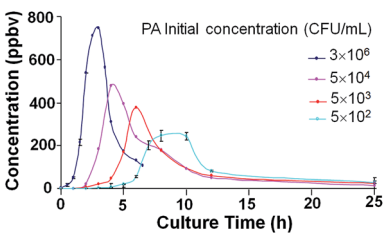

Fig. 4 Time profiles of 1-pyrroline signal (*m*/*z* 70) detected by AMS from the headspace of cultures with different initial concentration of PA.

3.7. Ionization mechanism of 1-pyrroline

The prevalence of 1-pyrroline over the rest of VOC signals in PA culture is very remarkable. The ionization of bacterial volatiles by corona discharge in our experiments most likely occurs *via* a similar mechanism to that in atmospheric pressure chemical ionization (APCI).[39] In APCI, analyte solution is pneumatically nebulized when passed through a heated capillary (>400 °C), and the aerosol cloud is subjected to atmospheric corona discharge. The majority of ion–molecule reactions in corona discharge area are gas-phase acid–base type reactions in which protons are gradually transferred along the ladder from chemicals with lower proton affinity – primarily from protonated water cations – to chemicals with higher proton affinity value.[39] This ladder proton transfer is very fast owing to the high rate of ion–molecule collisions at atmospheric pressure. As a result, thermodynamic equilibrium between reacting species is typically reached during ionization. 1-Pyrroline has a very high proton affinity value of 926 kJ mol^{-1}.[43] For comparison, proton affinity of water is 691 kJ mol^{-1}, proton affinity of ammonia is 854 kJ mol^{-1}, and proton affinity of pyridine is 930 kJ mol^{-1}.[43] Owing to its high proton affinity and high vapor pressure, 1-pyrroline acts as an efficient proton scavenger to suppress ionization of VOCs with lower proton affinity values. Similarly, the addition of highly basic pyridine or trimethylamine into sample solution is known to heavily suppress ionization of other analyte molecules. Apart from protonated 1-pyrroline, the only other signals observed at high intensity in AMS of PA corresponded to radical water cations (*m*/*z* 36 and *m*/*z* 54) formed *via* electron abstraction rather than protonation. Other signals were observed at a much weaker abundance. Therefore, ambient ionization by corona discharge can be considered as a highly sensitive and selective approach to detect 1-pyrroline in PA cultures.

Interestingly, earlier studies revealed the ubiquitous presence of protonated 1-pyrroline cations in the Earth troposphere.[43] The process of tropospheric ion formation proceeds *via* essentially the same charge-transfer ladder as described above. Initially, cosmic rays and radioactive decay in the troposphere initiate the production of protonated water cations. These primary water cations donate protons to the successively less abundant neutral species with higher proton affinity to form progressively more stable protonated cations. As a result, trace neutral compounds, such as pyridine and 1-pyrroline, can serve as precursors for relatively abundant tropospheric ions.[43] The ubiquitous observation of 1-pyrroline cations in the air illustrated the high chemical selectivity of corona discharge ionization toward 1-pyrroline and the high stability of protonated 1-pyrroline cations in the gas phase.

3.8. Analytical challenges of 1-pyrroline detection by other methods

Although the detection of 1-pyrroline in the volatile headspace of PA cultures is readily achieved using the ambient corona discharge ionization, it appears to be more challenging by many other methods. To our knowledge neither offline approaches (*e.g.*, gas chromatography mass spectrometry (GC-MS))[14,44,45] nor online methods (*e.g.*, selected ion flow tube mass spectrometry (SIFT-MS)[46] or secondary electrospray ionization mass spectrometry (SESI-MS)[36]) reported until today could reveal the presence of 1-pyrroline in PA or in other bacterial species. The detection of 1-pyrroline is complicated by its low chemical stability. In room-temperature aqueous solutions 1-pyrroline usually degrades on the time scale of several days,[47] and the degradation process is further accelerated at higher temperatures.[47,48] Therefore, significant degradation of 1-pyrroline could be expected if high temperatures are used for vapor collection and/or analysis, such as in GC-MS. Another problem associated with the poor chemical stability of 1-pyrroline is the lack of commercially available reference standard, which greatly complicates the unambiguous chemical assignment of 1-pyrroline signal. Finally, because 1-pyrroline is released transiently within the early period of incubation, the detection of 1-pyrroline is best achieved at relatively short incubation times. Therefore, too long incubation times in some studies could be another possible explanation for the lack of 1-pyrroline observation.[36] Our data demonstrate the APCI type of ionization is highly suitable for the sensitive MS detection of 1-pyrroline vapor. Importantly, vapors from the bacterial cultures are sampled to ionization at room temperature without additional heating. The mild sampling conditions protect the intact molecular structure of 1-pyrroline and may contribute to the high visibility of 1-pyrroline in our experiments. We expect that the detection of 1-pyrroline in the headspace of PA cultures should also be easily achieved using other types of ambient plasma ionization, *e.g.*, DART-MS[20] or LTP-MS.[22]

3.9. 1-Pyrroline metabolism in PA

1-Pyrroline is known to be released by some plants presumably for odor mimicking purposes[40,49] and by some animals presumably as pheromones.[50–52] However, the exact mechanism of 1-pyrroline formation by living organisms remains unknown. Below we mention a possible explanation for the high specificity of 1-pyrroline release by PA, even though experimental proof is lacking. 1-Pyrroline is a possible oxidation product, directly or indirectly, from at least 7 common metabolites, including pyrrolidine, proline, ornithine, 4-aminobutanol, putrescine, spermidine and spermine.[42] The generation of 1-pyrroline from putrescine by spontaneous atmospheric oxidation[42] or from proline by the Strecker degradation[53] was demonstrated in laboratory conditions. In living organisms, both proline and putrescine can mediate cellular response to abiotic stresses, such as cold and dehydration.[54,55] Therefore, the enhanced emission of 1-pyrroline during the lag phase of PA culture could possibly be related to the enhanced turnover of proline, putrescine and other structurally relevant molecules over the process of bacterial adaptation to growing conditions. The specific observation of 1-pyrroline emitted by PA cultures but not by the other strains might thereby reflect the mechanistic distinction of stress adaptation in *Pseudomonas* relative to other genera. The gradual decrease of 1-pyrroline level in PA cultures after the initial increase (Fig. 4) indicated that the produced 1-pyrroline was readily metabolized by bacteria into other products or degraded due to its low chemical stability. The short metabolic life-time of 1-pyrroline has also been suggested by earlier studies[52,56] and might be another factor complicating the detection of 1-pyrroline in PA cultures using offline methods.[14]

3.10. Identification of PA infection in clinical samples *via* targeted 1-pyrroline detection

PA infections are most frequently acquired by immunocompromised patients in hospitals and nursing homes, accounting for *ca.* 10% of hospital-acquired infections, causing pneumonia, urinary tract infection, bacteremia, surgical wound infection, osteomyelitis, *etc.*[1] Predisposing conditions for acquiring PA infection include hematologic malignancies, immunodeficiency relating to AIDS, neutropenia, diabetes mellitus, severe burns, and particularly cystic fibrosis. The lungs of cystic fibrosis patients can be chronically infected by PA for several years.[57] Clinical diagnosis of PA infection usually relies on microorganism identification in a clinical laboratory, which is laborious, time-consuming and carries a risk of misidentification.[3] Hence is the increasing attention to identification of microorganisms using MS approaches, which allow much higher molecular specificity of the analysis.[3–5]

Based on our findings, 1-pyrroline can be proposed as a volatile differential metabolite for the rapid identification of PA infections in humans using commonly available MS instrumentation. In a recent study, we analyzed blood cultures of 61 adult volunteers at suspicion of bacteremia and 39 patients confirmed with bacteremia.[38] Out of the studied cultures, 3 cases of bacteremia were assigned to PA infection through conventional microorganism identification in clinical laboratory for 2–3 days, and the AMS of the same cultures showed a notable signal at m/z 70 after few hours of incubation (0.5–6 h). Further, 30 blood cultures of healthy individuals inoculated with PA (10^4 CFU mL^{-1}) were tested by AMS, and each of them produced the

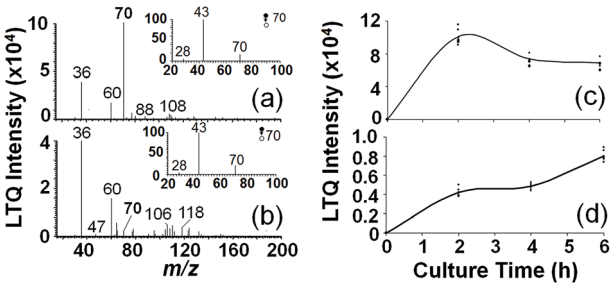

Fig. 5 AMS spectra of VOCs from a PA sputum culture (a) and PA urine culture (b) incubated for 2 h at the initial PA counts of 2×10^5 CFU mL^{-1} and 2×10^4 CFU mL^{-1} accordingly. Signal intensity of 1-pyrroline signal (m/z 70) is shown at four different incubation times (0 h; 2 h; 4 h; 6 h) for seven biological replicates (c and d).

m/*z* 70 signal upon incubation.[38] However, no conclusive structural assignment for *m*/*z* 70 was made in our preliminary work.[38]

In current study, using tandem analysis we confirmed that the *m*/*z* 70 biomarker signal in the blood cultures of PA patients indeed corresponded to protonated 1-pyrroline. The same approach was directly extended for the rapid identification of PA infections in urinary and respiratory tracts using the AMS analysis of urine and sputum cultures. 1-Pyrroline was clearly visible by AMS of the volatile headspace from seven sputum cultures and seven urine cultures incubated for 2 h at the initial PA count of 2×10^5 CFU mL^{-1} and 2×10^4 CFU mL^{-1} accordingly (Fig. 5a and b). The important feature of urine and sputum spectra was a relatively low inter-individual variability in MS signal intensity of 1-pyrroline, with RSD within 5–10% (Fig. 5c and d). In contrast, our earlier study showed pronounced inter-individual variability of 1-proline signal in simulated blood cultures.[38] Some blood cultures of PA did not reveal any bacterial growth even after 16 h of incubation. The considerably slower bacterial growth in the blood cultures of certain human individuals was attributed to possibly higher antibacterial blood immunity of those individuals.[38] Apparently, both urine and sputum have weaker bacterial resistance than blood, which could account for the much lower inter-individual signal variability. The low inter-individual signal variability is important for the quantitative evaluation of PA concentration in culture solution.

In this study we did not pursue quantitative measurements of PA concentration in urine and sputum cultures after incubation. For the accurate quantitative analysis of PA concentration based on 1-pyrroline AMS signal intensity one should definitely take into account matrix effects, most importantly those associated with solution pH. The pK_a value of 1-pyrroline is $\approx$6.7, which means that 1-pyrroline is half ionized at pH 6.7.[42] At higher pH values the relative share of nonionic 1-pyrroline will increase, resulting in the increase of molecular volatility. In contrast, at lower pH values the relative share of ionic 1-pyrroline will increase, resulting in the decrease of molecular volatility. The sensitivity of 1-pyrroline vapor pressure to solution pH should be particularly high around pH 6.7. Therefore, calibration curve for 1-pyrroline should be built in the same matrix as the clinical sample. To account for possible inter-individual differences of matrix composition, the method of standard additions can be recommended for the most accurate and reliable quantification of 1-pyrroline in patient cultures based on the AMS analysis of volatiles.

3.11. Analytical merits and limitations of VOC screening by AMS

The principal limitation of MS analysis compared to the traditional biochemical approaches is that different culture tubes are analyzed sequentially rather than simultaneously. Therefore, the speed of MS sampling is particularly important, because it limits the number of samples that can be measured during the available instrument time. VOC analysis by AMS is featured by the high speed and throughput of sampling. At present we can routinely achieve scanning rate of *ca.* 5 samples per min. This high throughput of sampling is achieved owing to the technical simplicity of experimental procedure and the efficient obviation of sample carry-over effects in VOC mode of analysis. We estimate that the sampling throughput can be further increased using robotized sampling procedure, similar to that in commercial GC instruments.

An important analytical merit of ambient corona discharge ionization is the total obviation of solvents during sampling and ionization of volatile chemicals. This allows for the low cost of operation and for the high stability of approach. When operated on a daily basis, the signal intensity of reference 1-pyrroline standard usually displayed relative deviations below 10%. Abrupt changes of signal intensity (>50%) were sometimes noticed upon instrument maintenance, in which case the calibration curve for 1-pyrroline (Fig. S-2†) was rebuilt to account for the change in instrument response.

4. Conclusion

In this study we discovered 1-pyrroline as a differential volatile metabolite of *Pseudomonas aeruginosa*. 1-Pyrroline emitted by PA features early release, high chemical specificity and excellent visibility by widely available MS instrumentation with atmospheric ion interface. The diagnostic value of 1-pyrroline was demonstrated for several clinical samples, including blood, urine and sputum. While common clinical diagnostic routines usually require *ca.* 2–3 days of analysis, our approach offers the same day diagnosis of PA, which allows earlier and more efficient disease treatment.

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