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Quantitative analysis of volatile metabolites released *in vitro* by bacteria of the genus *Stenotrophomonas* for identification of breath biomarkers of respiratory infection in cystic fibrosis.

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Abstract

The aim of the present study was to characterise the volatile metabolites produced by genotypically diverse strains of the *Stenotrophomonas* genus in order to evaluate their potential as biomarkers of lung infection by non-invasive breath analysis. Volatile organic compounds (VOCs) emitted from 15 clinical and five environmental strains belonging to different genogroups of S*tenotrophomonas maltophilia* (n=18) and *Stenotrophomonas rhizophila* (n=2) cultured in Mueller-Hinton Broth (MHB) liquid media were analysed by gas chromatography mass spectrometry (GC-MS) and selected ion flow tube mass spectrometry (SIFT-MS). Several VOCs were detected in high concentration, including ammonia, propanol, dimethyl disulphide, and dimethyl trisulphide. The GC-MS measurements showed that all 15 clinical strains produced similar headspace VOCs compositions and SIFT-MS quantification showed that the rates of production of the VOCs by the genotypically distinct strains were very similar. All *in vitro* cultures of both the *Stenotrophomonas* species were characterised by efficient production of two isomers of methyl butanol, which can be described by known biochemical pathways and which is absent in other pathogens including *Pseudomonas Aeruginosa* . These *in-vitro* data indicate that methyl butanol isomers may be exhaled breath biomarkers of *S. maltophilia* lung infection in patients with cystic fibrosis.

Introduction

The genus *Stenotrophomonas* encompasses Gram-negative, strictly aerobic bacteria which are ubiquitous in aqueous environments, soil and plants [1]. These organisms have also been used for biotechnology applications [2]. Although they are generally non-pathogenic to healthy people, they may cause serious infections in patients with severe underlying disease or impaired immunity. Risk factors associated with *Stenotrophomonas* infection include HIV infection, malignancy, cystic fibrosis, neutropenia, the presence of mechanical ventilation or central venous catheters, recent surgery, trauma, and therapy using broad-spectrum antibiotics. The number of *Stenotrophomonas* infections in humans has dramatically increased in recent years, especially in patients with cystic fibrosis (CF) [3]. The lack of mucociliary clearance in the airways of CF patients leads to the build-up of immovable mucus in the lungs and airways of CF patients provides a substrate for colonization by different bacteria, notably *Pseudomonas aeruginosa*, [4], *Staphylococcus aureus* and *Streptococcus pneumoniae* [5], *Stenotrophomonas* spp. with which the present paper is largely concerned, and the fungus *Aspergillus fumigatus* [6]. Early diagnosis and antibiotic treatment of such infections can improve the prognosis and extend the patients' life [7]. Some details of the genetic aspects of the genus *Stenotrophomonas* are given in Appendix 1 and the biochemical routes to the production of volatile organic compounds (VOCs) by this bacterium are given in Appendix 2.

Breath analysis holds promise as a non-invasive diagnostic of lower respiratory tract infections, obviating the routinely-used invasive procedures like bronchoscopy or alveolar lavage [8]. Our recent SIFT-MS studies of VOCs released by *in vitro* cultures of the bacteria *P. aeruginosa*, *S. aureus*, *S. pneumoniae*, and the fungus *A. fumigatus* [6], suggest that these VOCs may appear in exhaled breath of persons infected with these pathogens. The non-invasive breath analysis approach to diagnosis would be

of great value, especially in children with CF where early detection of respiratory pathogens increases the chance of eradication and improves prognosis [9].

Analyses of volatile compounds (VOCs) emitted from *Stenotrophomonas* bacterial cultures *in vitro* is a natural extension of our on-going search for volatile biomarkers of lung and airways infections by breath analysis. Selected ion flow tube mass spectrometry (SIFT-MS) is being successfully used for on-line, real time quantification of trace gases in human breath [10, 11] and has great potential as a tool for non-invasive physiological monitoring of CF patients [4, 9]. A notable recent advance is the identification of HCN as a biomarker of *P. aeruginosa* in CF patients; studies based on SIFT-MS have shown that both HCN [4, 9] and methylthiocyanate [12] are specific volatile biomarkers of this pathogen. SIFT-MS has been recently exploited to quantify a range of volatile metabolites released by various *in-vitro* cultures of *P. aeruginosa* [13] and other respiratory pathogens [5, 6]. Similarly, gas chromatography-mass spectrometry (GC-MS) is a well-established technique for untargeted analyses of volatile compounds in complex matrices and has been exploited to identify many of the characteristic VOCs released by bacterial cultures [14, 15].

The present study aimed to assess the VOCs released by *in vitro* cultures of *Stenotrophomonas* spp. in order to identify potential biomarkers of infection of the airways by these bacteria in CF patients via breath analysis. This work, first presented at the Breath Analysis 2014 meeting in Torun, Poland, has been conducted using the powerful combination of GC-MS and SIFT-MS by which VOCs released by a genotypically characterized bacterial collection encompassing 18 strains of *S. maltophilia* isolated from clinical (n=15) and environmental (n=3) specimens and two strains of *S. rhizophila* of environmental origin have been investigated.

Materials and methods

Origin and genetic diversity of the bacterial strains

The study set encompassed 20 *Stenotrophomonas* strains, including 15 *S. maltophilia* strains isolated from clinical specimens and five environmental strains (three *S. maltophilia* and two *S. rhizophila*) from natural environments (Table 1). Nine of the 15 clinical strains were recovered from the respiratory specimens of CF patients at the Cystic Fibrosis Centre in Prague. The taxonomic affiliation of these 20 strains were determined previously [16]. The previous work using multi-locus sequence typing (MLST) has delineated nine different genogroups (GGs) [17]. The clinical strains included in the present study belong to at least four of these genogroups (GGs) with GG6 containing most of them. All five environmental strains belong to either the strictly environmental species *S. rhizophila* (previous GG8) or GG9 [18-20].

Table 1. The 20 *Stenotrophomonas* strains studied

Strain*	Species (genogroup)#	Specimen	Year of isolation
CNCTC 5821^T	*S. maltophilia* (GG6)	Orypharynx	Before 1964
ANC 4262 (= Cf1)	*S. maltophilia* (GG6)	CF sputum	2004
ANC 4263 (= Cf2)	*S. maltophilia* (GG6)	CF throat swab	2005
ANC 4264 (= Cf3)	*S. maltophilia* (GG6)	CF sputum	2005
ANC 4265 (= Cf4)	*S. maltophilia* (GGnew)	CF sputum	2005
ANC 4266 (= Cf5)	*S. maltophilia*	CF sputum	2005
ANC 4267 (= Cf6)	*S. maltophilia* (GG6)	CF sputum	2006
ANC 4268 (= Cf7)	*S. maltophilia* (GG6)	CF sputum	2006
ANC 4269 (= Cf8)	*S. maltophilia* (GG1)	CF sputum	2007
ANC 4331 (= Cf9)	*S. maltophilia* (GG6)	CF sputum	1989
ANC 4332 (= Cli4)	*S. maltophilia* (GGnew)	Blood culture	1994
ANC 4333 (= Cli5)	*S. maltophilia* (GG4)	Endotracheal tube	1994
ANC 4336 (= Cli2)	*S. maltophilia*	Venous catheter	1993
ANC 4338 (= Cli7)	*S. maltophilia* (GG6)	Wound	1997
ANC 4339 (= Cli8)	*S. maltophilia* (GGnew)	Blood	1997
ANC 4341 (= Env1)	*S. maltophilia* (GG9)	Meadow	2007
ANC 4342 (= Env2)	*S. maltophilia* (GG9)	Meadow	2007
ANC 4343 (= Env11)	*S. maltophilia* (GG9)	Cave	2010
ANC 4344 (= Env13)	*S. rhizophila* (GG8)	Cave	2010
ANC 4345 (= Env14)	*S. rhizophila* (GG8)	Cave	2010

[*] Strain codes used by Nurvar et al. [16] are in parentheses.
[#] Assignation to genogroup based on partial *gyrB* gene sequence analysis [17].

Culturing and quantification of bacteria

Liquid (submersion) cultures of each strain were prepared in 7 mL of Mueller-Hinton Broth (MHB) (Oxoid Ltd, UK) in 100 mL hermetically sealed flasks [12]. These cultures were inoculated with ~50 µL of cell suspension of standardized turbidity (~10^8 colony forming units [CFU] mL^{-1}) prepared in saline from an overnight agar culture, and these were cultured with continuous stirring in a thermostatically controlled water bath for 20 h. The cultures of the 15 clinical strains were grown at 37°C whereas the cultivation temperature for the five environmental strains had to be lowered to 30°C because of insufficient growth at 37°C. As a control, sterile liquid MHB medium cultured under the same conditions was included in all experiments. All strains and controls were processed in duplicate within each experiment. Bacterial growth was assessed both by CFU counting and by optical density measurement. The CFU mL^{-1} of all strains cultured for 20 h ranged from $0.9x10^{10}$ to $1.9x10^{10}$.

SPME headspace sampling protocol and GC-MS analysis.

VOCs were extracted from the headspace of the bacterial cultures using solid phase microextraction (SPME) onto carboxen/polydimethylsiloxane (CAR/PDMS)-coated fibres (Supelco, Bellefonte, PA, USA) for 30 min at a temperature 37°C. Immediately following the extraction period, the SPME fibres were directly inserted into the injector of the GC-MS instrument (FOCUS GC with SSL, ITQ 700 ion trap mass spectrometer using electron ionisation) held at 210°C. The GC conditions were the following: splitless injection, He carrier gas at 1 mL/min, GC oven temperature program 38°C (hold 13 min) 3°C/min ramp up to 100°C (hold 5min), 4°C/min to 150, 20°C/min to 210°C and a final hold 3 min (total run time 58 min). A GC-MS capillary column TG-624 (fused 100% Cyanopropylphenyl Polisiloxane, 30m x 0.25mm ID x 1.0um film) was used. Electron ionisation at 70eV generated ions that

were analysed by the ion trap operating in the scan mode (*m/z* 15–400, scan rate 1 scan/s). Peak identification was based on mass spectral interpretation and comparisons with the NIST 2.0 library [21]. Kovats retention indices were obtained by analysing standard mixtures of different C_5-C_{10} hydrocarbons. Whilst the SPME/GC-MS technique is a suitable method for identification of unknown molecules in complex matrices, quantification of compounds is time consuming and not precise. SIFT-MS is able to accurately quantify specific VOCs in complex mixtures in real time and so the combination of SPME/GC-MS and SIFT-MS is powerful for the analysis of complex mixtures.

SIFT-MS analysis

The SIFT-MS analytical technique has been described in detail previously [10, 11, 22] and the particular aspects of its use to analyse *in vitro* bacterial cultures are detailed in the previous papers by Shestivska et al [12, 13]. Thus, the headspace of the bacterial cultures was analysed using judiciously chosen reagent ions (H_3O^+, NO^+ or O_2^+) to produce analyte ions that identify the neutral trace compounds present in the headspace [10]; the the count rates of these analyte ions provide accurate absolute quantification of the precursor neutral trace compounds down to concentrations as low as parts-per-billion by volume, ppbv [23-25]. A list of compounds quantified by SIFT-MS, together with the reagent ions and the analyte ions used for their identification and quantification, is given in Table 2. Some of the compounds identified by GC-MS analyses were specifically targeted for SIFT-MS analysis. The data obtained were assessed by principal component analysis (PCA) using the Statistica software (StatSoft Czech Republic s.r.o.), as explained and discussed later.

Table 2. Reagent and product ions used for SIFT-MS quantification of volatile metabolites in the headspace of cultures of the 20 *Stenotrophomonas* strains. Note that SIFT-MS quantifications correspond to the sum of concentrations of all isomers of the compounds indicated.

SIFT-MS	MW g/mol	reagent ion	Analyte (product) ions m/z
Methanol	32	H_3O^+	33, 51, 69
Propanol	60	H_3O^+	43
Butanol	74	H_3O^+	57, 75, 93
Methyl butanol	88	H_3O^+	71, 89, 107
Hydrogen sulphide	34	H_3O^+	35, 53
Methanethiol	48	H_3O^+	49, 67, 85
Dimethyl sulphide	62	O_2^+	62, 80
Dimethyl disulphide	94	NO^+	94
Butanone	72	NO^+	102
Ammonia	17	O_2^+	17, 35
Methyl benzoate	136	H_3O^+	137
Pentane	72	O_2^+	42, 72
Butyric acid	88	NO^+	71, 118

Results and discussion

Sample chromatograms obtained by headspace SPME/GC-MS analyses of cultivated sterile medium and of identical media cultivated with clinical and environmental *S. maltophilia* strains are shown in Fig. 1. The GC-MS data show that the qualitative composition of the produced VOCs (see Table 3) is very similar for all 20 *Stenotrophomonas* strains studied.

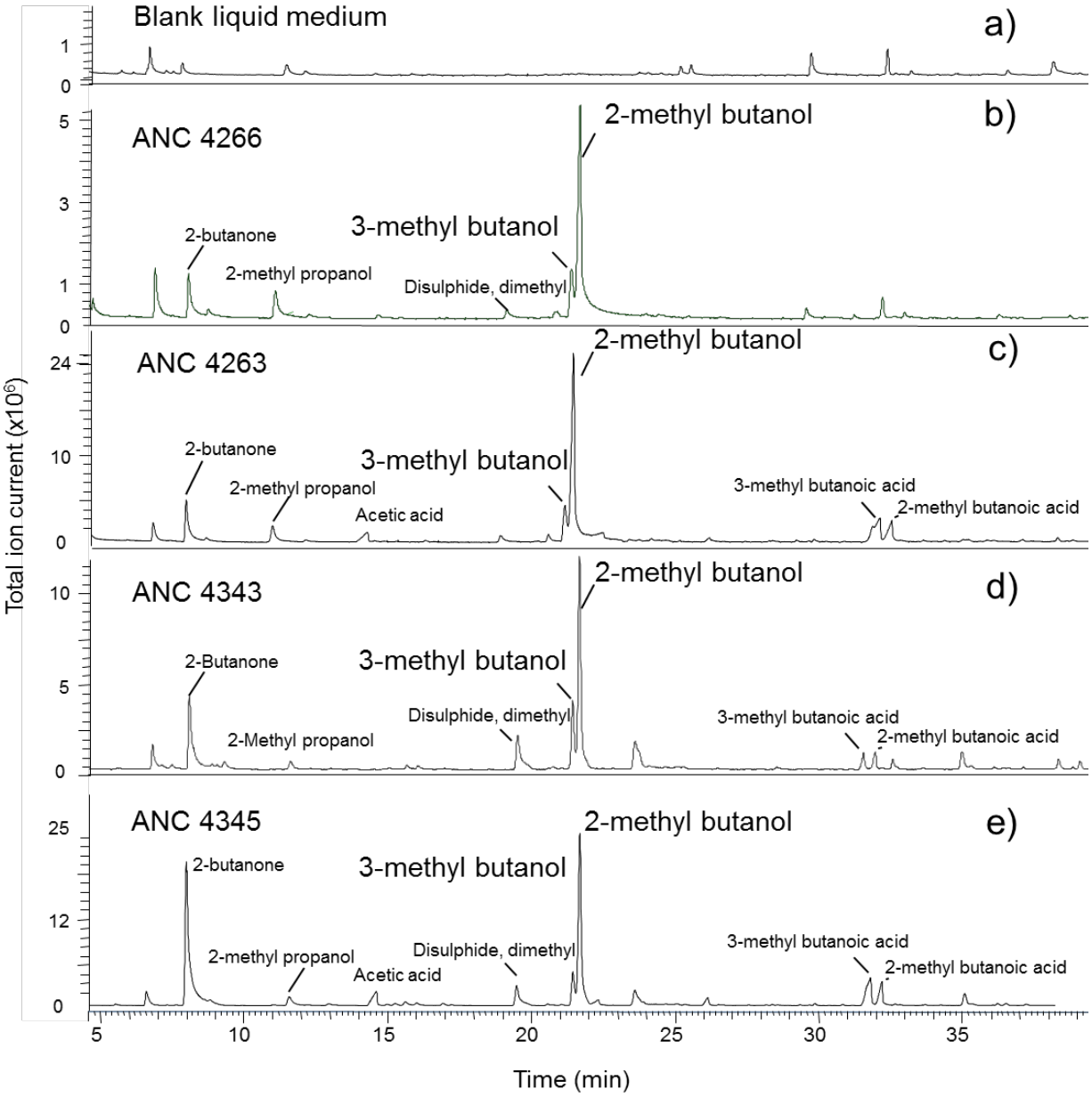

Fig. 1. GC-MS analyses of VOCs extracted using SPME from the headspace of a) blank sample (liquid MHB medium), b) identical medium cultivated with the clinical *S. maltophilia* strain ANC 4266 c**)** clinical *S. maltophilia* strain ANC 4263 (GG6), d) environmental *S. maltophilia* strain ANC 4343(GG9), e) environmental *S. rhizophila* strain ANC 4345 (GG8).

The SIFT-MS analyses provided absolute concentrations of 13 VOCs given in Fig. 2, but were unable to distinguish the two methyl butanol isomers (both with a molecular weight of 88). SIFT-MS indicated presence of relatively high concentrations of ammonia (500 to 2000 ppbv), which is commonly produced by bacteria, including oral cavity bacteria, and so it cannot be used as a reliable volatile

biomarker of lung infection. However, its elevation in the breath of patients may be a useful co-biomarker in combination with other specific volatiles such as methyl butanol (see below).

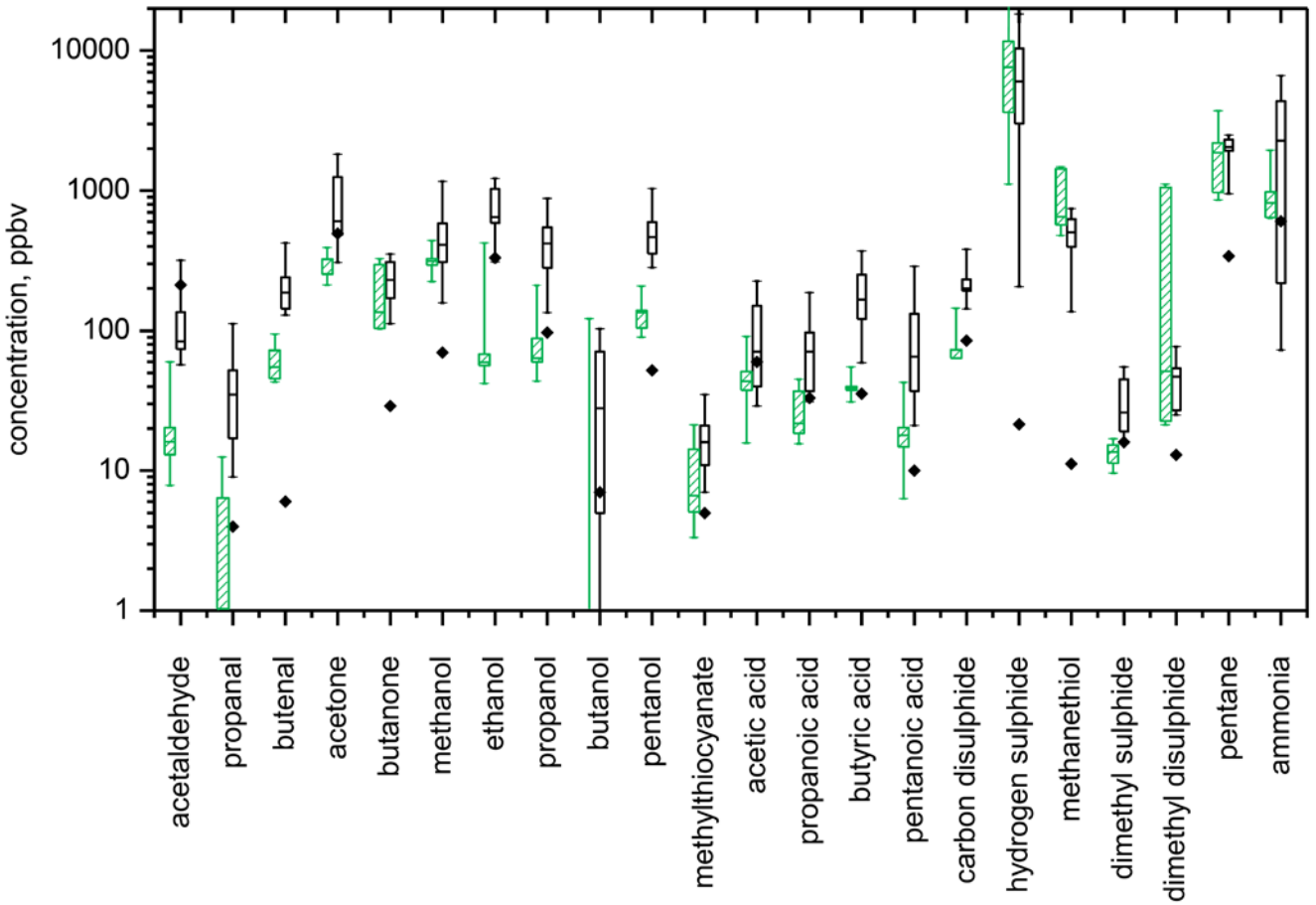

Fig. 2. SIFT-MS quantifications (in ppbv) of volatile metabolites detected in the headspace of cultures of the 20 *Stenotrophomonas* strains indicated by box and whiskers plots; dashed green for environmental and open black for clinical strains (whiskers indicate minimum and maximum range, boxes indicate 25th and 75th percentile and the lines indicate medians). The diamond data points show median concentrations in the headspace of cultured control medium.

Table 3 shows the VOCs identified by SPME/GC-MS in the culture headspace of the 20 *Stenotrophomonas* strains. Fourteen of these VOCs were seen in all the strains, including acetone, 2-

butanone, 2-propanol, dimethyl disulphide, 2- and 3-methyl butanol. These VOCs can also be released by other bacterial cells, but in different concentration, as previously reported [5, 6, 12, 13].

Table 3. Volatile metabolites detected by SPME/GC-MS the headspace of cultures of the 20 *Stenotrophomonas* strains. Peak areas corresponding to the headspace concentrations of selected VOCs are indicated as percentages of observed maximum as: ↓<25%, ○=25-75% and ●>75%.

Compound	Retention time (m)	Retention index	Medium only	Clinical	Environmental
1-propanol	7.03	616	↓	○	○
2-propanol	4.44	547	○	○	○
2-butanol	9.38	650	↓	○	●
2-methyl-1-propanol	12.23		↓	●	●
1-pentanol	25.00	833	↓	○	◌
3-methyl butanol	22.48	800	↓	●	●
2-methyl butanol	22.76	804	↓	●	●
dimethyl disulphide	20.42	779	↓	○	●
dimethyl trisulphide	38.04		↓	○	○
methyl thiolacetate	16.40	737	↓	◌	○
2-butanone	8.53	638	○	●	●
2-pentanone	16.80	741	↓	○	○
2-heptanone	32.48	934	↓	○	○
butyric acid	29.04	885	↓	○	○

Several organosulphur compounds were identified in the headspace of both the clinical and environmental *Stenotrophomonas* strains. Some of these compounds are commonly generated by bacteria, including those often present in the healthy human oral cavity responsible for oral malodour (hydrogen sulphide, methanethiol) [26-28]. However, one sulphur compound, dimethyl trisulphide, which was identified by SPME/GC-MS in the headspace of all 20 strains (see Table 2), is not usually released by non-*Stenotrophomonas* bacteria [4, 5] and, thus, may be considered as a potential volatile biomarker in exhaled breath specific to *Stenotrophomonas* infection.

Several aldehydes, including methyl butanal, were observed in the headspace of the blank MHB liquid media and their concentrations decreased in the headspace of bacterial cultures; thus, they are not included in Table 2 and Table 3. This removal of aldehydes from culture media by bacterial and some mammalian cells has been observed in previous studies [29-32].

Presence of methyl butanol isomers in the headspace of Stenotrophomonas cultures

The SPME/GC-MS data show similar qualitative VOCs composition for all 20 strains. As examples of positive compound identification, total ion current chromatograms obtained for the sterile MHB liquid medium and four selected *Stenotrophomonas* strains are shown above in Fig.1. An important observation is that all the strains produced high amounts of two isomers of methyl butanol (Fig.1) (retention times 20.72 min for 3-methyl-1-butanol and 21.04 min for 2-methyl-1-butanol), so we have given special attention to these compounds. The elution times and the ionisation fragmentation patterns of these two compounds agreed with those of reference standard sample of these isomers and also with those included in the NIST ion mass spectral library NIST 2.0 [21]. Methyl butanol was observed in all the 20 strains (ranges of ion signals: $1.8x10^5$ to $2.5x10^7$ for 3-methyl-1-butanol and $3.2x10^7$ to $2x10^8$ for 2-methyl-1-butanol), the ion signal level of 3-methyl-1-butanol being typically 20% of that for 2-methyl-1-butanol, as indicated by the slope of the plot in Fig. 3.

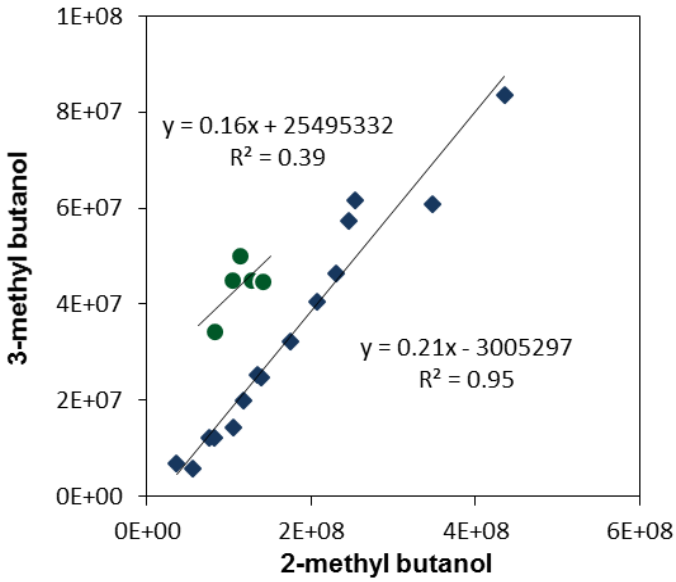

Fig. 3. GC-MS peak area correlation between 2-methyl butanol and 3-methyl butanol detected in the headspace of 20 *Stenotrophomonas* strains cultured in liquid MHB medium. The diamonds indicate the 15 clinical strains and the circles indicate the 5 environmental strains. Note the different correlations (R^2 values) for the two types of strain (see the text).

Fig. 3 also indicates the close correlation (more than 80%) between the GC-MS signal peak areas for 2- and 3- methyl butanol generated by the 15 clinical *Stenotrophomonas* strains. The analogous correlation for the five environmental strains is weaker (R^2= 0.39). This can be related to different metabolisms of those two groups of bacteria, but given the small number of samples this weaker correlation should not be over interpreted. Note that SIFT-MS quantification provides the total concentration of these two butanol isomers and their pentanol isomers; but, according to these GC-MS data, pentanol was present in the headspace of these bacteria strains in much smaller amounts (range of ion signals $<2x10^5$) compared to the methyl butanol isomers (see above).

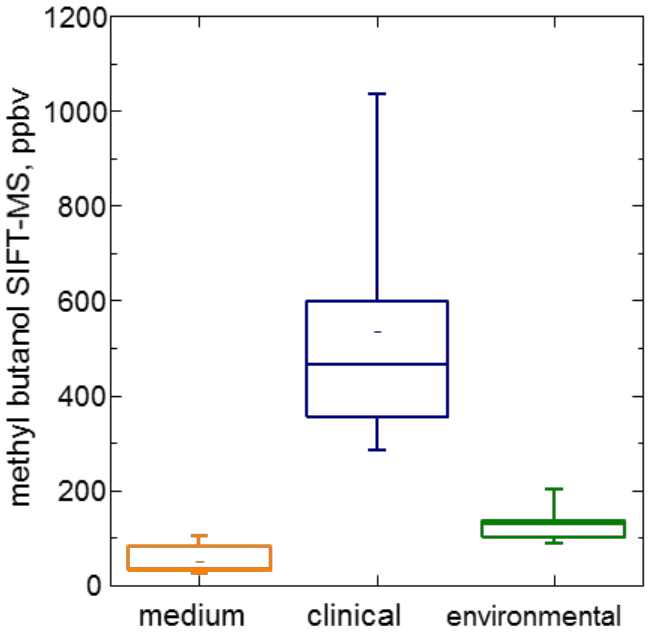

Fig. 4. Concentrations (ppbv) of total methyl butanol measured using SIFT-MS obtained for the headspace of 15 clinical and 5 environmental *Stenotrophomonas* strains cultured in liquid MHB medium. Also included, for comparison, are the results from identically cultivated medium samples that were not inoculated.

SIFT-MS quantification of total methyl butanol (sum of the concentrations of the two isomers) showed that these compounds were produced in the headspace of all 20 strains in significant concentrations within the range 87-1036 parts-per-billion by volume (ppbv). As shown by the box and whisker plots in Fig. 4, there is a clear difference between the concentrations of total methyl butanol in the headspace of the environmental strains (from 87 to 201ppbv) and the clinical strains (from 283 to 1036 ppbv.

Some clinical strains of *S. maltophilia* (genogroups other than GG9) produced much more methyl butanol than the environmental *S. maltophilia* (GG9) and *S. rhizophila*. This difference may result from the genetic distinction of the two bacterial species and different genogroups of *S. maltophilia*. If this is the case, such a difference could assist diagnosis of lung/airways infection by different *Stenotrophomonas* genogroups via breath analysis. However, more strains must be analysed using different culture media

before any definite conclusion can be drawn on this issue. In addition, the quantitative differences in the production of methyl butanol between the two bacterial groups may also reflect the different cultivation temperatures for the clinical (37°C) and environmental (30°C) strains that were needed to obtain approximately equal culture densities for all strains.

It is also important to note that methyl butanol has also been identified in the headspace of cultures of the major CF pathogen *P. aeruginosa* when cultured in MHB liquid medium [33, 34], but at much lower concentrations (within the range 1 -15 ppbv) [13]. This may further support the role of methyl butanol as a specific biomarker of *Stenotrophomonas* infection. Again, this assumption needs to be verified by more extensive studies.

Only a very weak correlation (R^2=0.07) is observed between the GC-MS peak area for methyl butanol and the total concentration of methyl butanol isomers as quantified by SIFT-MS. This is most probably due to the uncertainty in defining GC-MS peak areas and the variability of the extraction efficiency of volatile metabolites by the SPME fibre. Fig. 5 shows a comparison of the total GC-MS peak areas for methyl butanol and butanol in the headspace of all 20 *Stenotrophomonas* strains. The reasonable correlation (R^2 = 0.6) may suggest common biochemical origins for these isomers (see Appendix 2).

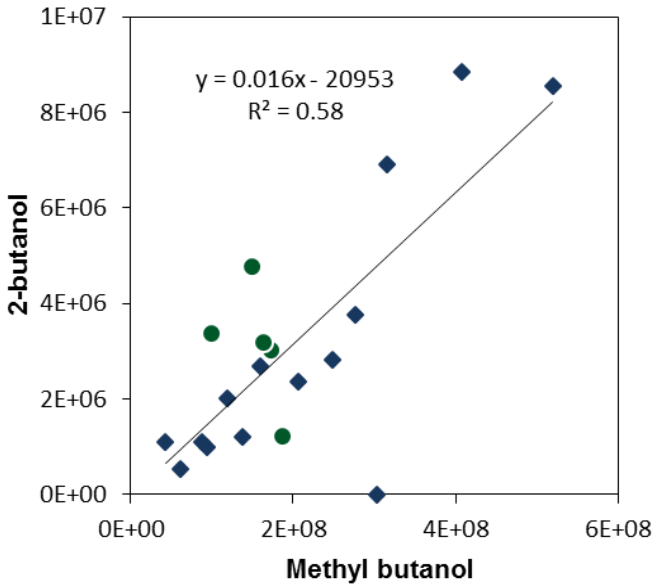

Fig. 5 GC-MS peak area correlation for total methyl butanol and 2-butanol detected in the headspace of 20 *Stenotrophomonas* strains cultured in liquid MHB medium. The diamonds indicate the 15 clinical strains and the circles indicate the 5 environmental strains.

Principal component analysis (PCA) of SIFT-MS concentrations data

Principal component analysis (PCA) methods, as used previously for the assessment of bacterial culture headspace data obtained using SIFT-MS [5, 35], have also been used in the present work to visualize the differences in the composition headspace concentration of VOCs emitted by the clinical and environmental *Stenotrophomonas* strains cultured in the same medium. The plot of the PCA scores of the first two principal components shown in Fig. 6 reveals a clear discrimination between the concentrations of volatile metabolites present in the headspace of clinical and environmental *Stenotrophomonas* bacterial cultures and culture medium alone. The main compounds important for discrimination between the environmental and the clinical strains are acetaldehyde, aminoacetophenone, ammonia, carbon disulphide and dimethyl disulphide, as indicated by the directions of the eigenvectors plotted in Fig. 6.

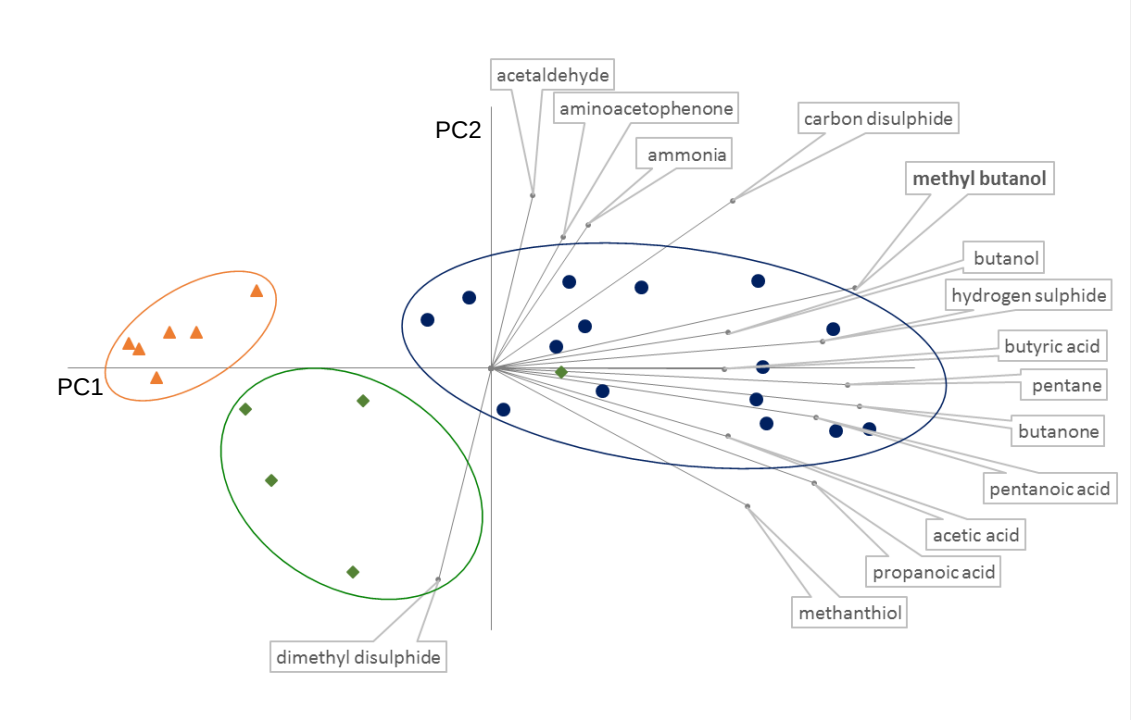

Fig 6. Principal component analysis (PCA) for SIFT-MS data obtained from full spectral scans. Scores of the first two principal components PC1 and PC2, which correspond to headspace concentrations, are indicted by **blue circles** for the 15 clinical strains, **green diamonds** for the 5 environmental strains and **orange triangles** for identically cultivated medium samples that were not inoculated. The lines indicate the projected directions corresponding to the individual compounds indicated. The PC1 scores plotted on the horizontal axis explain 29.5% of the variation and the PC2 scores plotted on the vertical axis explain 13% of the total variation.

Concluding remarks

The results of this combined SPME/GC-MS and SIFT-MS study indicate that methyl butanol is produced by both environmental and clinical strains of the genus *Stenotrophomonas* cultured *in vitro*, and that clinical strains produced methyl butanol in significantly higher concentrations compared to strains from the natural environment. Thus, methyl butanol might be present in measurable concentration in the exhaled breath of CF patients; if so, this could provide a specific and valuable non-invasive VOC diagnostic of early lung infection by *Stenotrophomonas*. Note that methyl butanol was not reported in previous studies of pathogens relevant to CF, viz. *P. aeruginosa*, *S. aureus*, *S. pneumoniae*, and the fungus

A. fumigatus [6]. More extensive work is needed to investigate this tentative postulate, including further *in vitro* bacterial culture studies as well as *in vivo* studies of the exhaled breath of precisely defined clinically and epidemiologically cohorts of patients. Also, it is important to keep in mind that the suggested method of SIFT-MS quantification of methyl butanol relies on the use of the analyte ions at *m/z* 71, 89 and 107 formed by its reactions with the H_3O^+ reagent ions. Whilst currently no overlaps with common breath metabolites are known at these *m/z* values, this cannot be entirely ruled out.

This study again shows that by direct analysis of the headspace of active bacterial cultures by SIFT-MS, absolute concentrations of several volatile metabolites can be obtained simultaneously. Reporting of absolute concentrations is most valuable in such *in vitro* experiments when the ultimate objective of the work is to provide guidance to envisaged *in vivo* investigations and the anticipated exploitation of breath analysis for the detection bacterial infection in the airways and lungs. Diagnosis of airways infection by sample (sputum, lavage) collection is often challenging, especially in children, and so immediate non-invasive diagnosis by the identification and quantification in exhaled breath of volatile biomarkers of specific infections is very attractive. Such could provide a rapid identification of the infecting bacterium, thus indicating more appropriate treatment, and a concomitant reduction of stress for both patients and clinicians alike.

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Appendix 1 Genetic diversity of the genus *Stenotrophomonas*

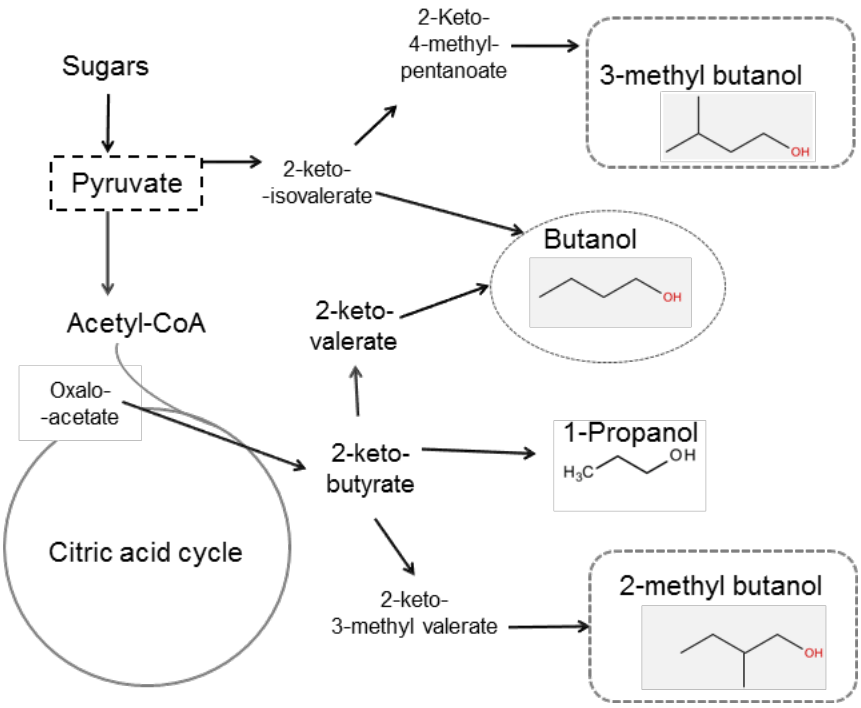
The genus *Stenotrophomonas* currently comprises 12 distinct species with valid names (http://www.bacterio.net/stenotrophomonas.html), but the genetic diversity is remarkable and further differentiation at the species level is expected [36]. This applies also to the most important species, *Stenotrophomonas maltophilia*, which has been only broadly defined and may represent several species according to the current taxonomic criteria. A number of genotypic methods have been used to unravel the taxonomic and phylogenetic diversity of *S. maltophilia*, including AFLP fingerprinting [37], 16S rRNA gene comparative analysis [20] and multilocus sequence typing (MLST) [17]. Most strains of *S. maltophilia* are characterized by their resistance to many currently available broad-spectrum antimicrobial agents [38]. The environmental strains of *S. maltophilia* show high resistance to antibiotics, as do the strains isolated from the sputum of CF patients, and the resistance profile pattern of the strains is seen to be independent of their source of isolation [39]. Many strains are susceptible to co-trimoxazole and ticarcillin, though resistance to these antibiotics has been increasing [40].

Strains from AFLP group 8 (= MLST genogroup (GG) 8 = 16S rRNA group E1) were recently accommodated in a novel species, *Stenotrophomonas rhizophila* [19]. This species has been isolated from environmental sites only. Another distinct lineage, AFLP group 9 (MLST genogroup GG9 = 16S rRNA group E2), also seems to be a strictly environmental group [18-20]. In contrast, the AFLP group 6 (= MLST genogroup GG6) has repeatedly been shown to be prominent among clinical strains [16, 17, 37]. The defining characteristics of *S. rhizophila* are as follows: growth at 4 °C and the absence of growth at 40 °C; the utilization of xylose as a carbon source; lower osmotic tolerance, the absence of lipase and *β*-glucosidase production and antifungal activity [41]. It has been observed that *S. rhizophila*, as a plant-associated bacterium, has the ability to grow at lower temperatures. As the environmental strains have a preference for lower temperatures, this might be related to their ability to survive in the rhizosphere.

Appendix 2 Biochemical routes to the production of methyl butanol by the genus *Stenotrophomonas*

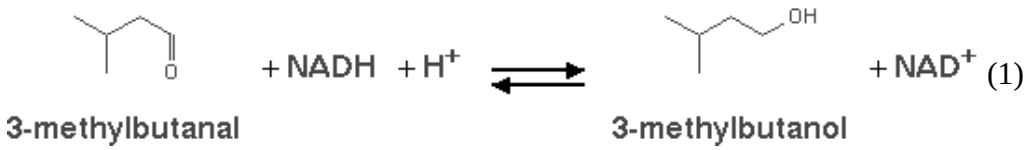
There are several known mechanisms of production of butanol and methyl butanol by bacteria. For example, butanol (biobutanol) can be naturally synthesized by solventogenic bacteria of the genus *Clostridium* through renewable substrates such as glucose. Higher-order alcohols (like 3-methyl butanol) can be produced through the ketoacid pathways [42, 43]. Scheme I describes possible and natural fermentative pathways for biosynthesis of branched-chain higher alcohols, including methyl butanol. This shows that metabolic synthesis of both methyl butanol and butanol is complementary. It is perhaps significant that both of these VOCs have been identified in measurable concentration in the headspace of the cultures of all 20 strains included in the present study , and that their headspace concentrations are well correlated, as seen in Fig. 5, probably indicating that they both originate from the keto-acid metabolic pathway (see Scheme I). Much previous research has been concerned with the biotechnology of methyl butanol production as a potential biofuel and so some details of the mechanism of methyl butanol production by different bacteria and yeast cells are already understood. A high yield for iso-butanol production from glucose by microbial fermentation has been achieved [44].

Scheme I shows that the two isomers of methyl butanol each originate from a different metabolic pathway: 3-methyl butanol from that proceeding via pyruvate and 2 methyl butanol from that proceeding via oxaloacetate, whilst butanol is produced in both of these pathways. The clinical and environmental strains included in the present study are genetically different and thus could produce different amount of enzymes.



Scheme I Metabolic keto-acid pathway for the biosynthetic production of 3-methyl butanol and 2-methyl butanol [42, 44]

Another possible synthesis of methyl butanol is from methyl butanal**,** even though is not considered to be physiologically relevant:



Scheme II

However, the correlation between methyl butanol and methyl butanal concentrations as measured in the present study of *Stenotrophomonas* is weak (R^2= 0.16). Thus, we judge that this metabolic pathway for methyl butanol production by this bacterium is unlikely.

S. maltophilia and S. rhizophila are ubiquitous in aqueous environments and soil. As organisms living in a wild environment, *Stenotrophomonas* have to coexist with many other bacteria and fungi that can produce toxic secondary metabolites. Thus, the competition between microorganisms and *Stenotrophomonas* bacteria for these ecological niches is very intense. *Stenotrophomonas* is also often associated with plants and has been isolated from rhizosphere in which wheat, oat, cucumber, maize, oilseed rape, and potato are grown. Consequently, *Stenotrophomonas* bacteria must have some specific strategies to exist in such environments and to manage the symbiotic relationship [45]. It is often a dominant member of the rhizosphere microbial community of plants and can produce high amounts of the plant growth hormone indole-3-acetic acid [39]. On the other hand, some studies suggest that *Stenotrophomonas* might potentially produce phytotoxic substance at one of stages of its life cycle, [46] causing root shortening due to 2-methyl butanol, 3-methyl butanol and 3-methyl butanal. Thus, we speculate that the high production of methyl butanol by *Stenotrophomonas* could be a specific genetically-conditioned pathway process in *Stenotrophomonas* bacteria.

From the evolutionary point-of-view, it is worth noting that methyl butanol production was detected in both the clinical and environmental *Stenotrophomonas* strains. Many studies have consistently shown that the environmental genogroups GG9 (syn. E2) and GG8 (*S. rhizophila*, syn. E1) branch at the base of *Stenotrophomonas* phylogenetic trees, and can therefore be considered ancestral lineages [18-20]. Thus, it can be inferred that the metabolic pathways leading to methyl butanol production were fixed early during *Stenotrophomonas* evolution before the divergence of pathogenic lineages (typically GG6). However, cellular metabolism of *Stenotrophomonas* is very complex and it is therefore difficult to account for every detail of the synthesis of methyl butanol by this bacterium. For effective optimization, details on the genes and enzymes responsible for the synthesis of the target compound, as well as undesired side products, need to be known.

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