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FEMS Microbiology Letters 220 (2003) 67–73

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Novel pyrazine metabolites found in polymyxin biosynthesis by *Paenibacillus polymyxa*

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Received 11 November 2002; received in revised form 12 January 2003; accepted 14 January 2003

First published online 1 February 2003

Abstract

A complex mixture of methyl-branched alkyl-substituted pyrazines was found in the growth medium of the polymyxin-producing bacterium *Paenibacillus polymyxa*, and of these, seven are new natural compounds. A total of 19 pyrazine metabolites were identified. The dominant metabolite was 2,5-diisopropylpyrazine as identified using a combination of high-resolution mass spectrometry, ^{1}H- and ^{13}C-nuclear magnetic resonance, gas chromatography-mass spectrometry as well as co-elution with an authentic standard. Its biosynthesis was correlated with growth and production was strongly stimulated by valine supplementation. The other pyrazine metabolites, all related pyrazines with either one, two or three alkyl substituents, were identified by means of their mass spectral data and/or co-elution with authentic standards.

Keywords: Pyrazine metabolite; New natural compound; *Paenibacillus polymyxa*

1. Introduction

Pyrazines are heterocyclic nitrogen-containing compounds found mainly in processed food, where they are created chemically in a dry heating process. They are also found naturally in many vegetables [1,2] and some microorganisms are known to produce pyrazines during their primary [3] or secondary metabolism [4]. Some pyrazines exhibit bactericidal [5] or chemoprotective [6] activities. In addition, many alkylated and methoxylated pyrazines exhibit strong odorous properties and are important flavouring compounds in a variety of food products [7]. Fig. 1 shows the general structure of a pyrazine molecule and indicates the four hydrogen atoms at the 2-, 3-, 5- and 6-positions that may be substituted by one to four alkyl, hydroxy or methoxy groups. Some of the best known naturally found pyrazines are 2-methoxy-3-isopropylpyrazine, sec-butyl- and isobutyl-substituted pyrazines from green peas (*Pisum sativum*) [1,2], 2-methoxy-3-isopropylpyrazine [8,9] and 2-methoxy-3-sec-butylpyrazine [10] as metabolites of *Pseudomonas* spp., 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 2,3,5,6-tetramethylpyrazine isolated from *Bacillus* species [3,11,12]. Occasionally, hydroxylated pyrazines are found, such as 2-hydroxy-3,6-diisobutylpyrazine-1,4-oxide (pulcherriminic acid) isolated from *Candida pulcherrima* [13], and 2-hydroxy-3-isobutyl-6-sec-butyl pyrazine-1-oxide (aspergillic acid) and 2-hydroxy-3-isobutyl-6-(1-hydroxy-1-methylpropyl) pyrazine-1-oxide (hydroxyaspergillic acid) isolated from *Aspergilli* [14].

Two different pathways for the biosynthesis of alkyl-substituted pyrazines have been proposed [1,8,13,14]. In one pathway the pyrazine ring structure is formed by amidation of an amino acid, followed by the condensation of the amidated amino acid with an α,β-dicarbonyl compound. A subsequent methylation reaction leads to alkyl- and methoxy-substituted pyrazines. The pathway was proposed for the synthesis of 2-methoxy-3-isopropylpyrazine, 2-methoxy-3-isobutylpyrazine and 2-methoxy-3-sec-butylpyrazine in *P. sativum* via condensation of glyoxal [1] or glyoxylic acid [2] with valine, leucine or isoleucine, respectively [1].

Other investigators suggested a pathway where the initial step in pyrazine biosynthesis is the condensation of two amino acids to a cyclic dipeptide, which is then converted into the pyrazine molecule [8,13,14]. Suggestions on this pathway include the biosynthesis of pulcherriminic

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0378-1097/03/$22.00
doi:10.1016/S0378-1097(03)00054-5

acid from the condensation of two L-leucine molecules to cyclo-L-leucyl-L-leucyl, which is then converted into pulcherriminic acid [13]. Analogous condensation reactions of leucine with isoleucine or valine with glycine are supposed to lead to hydroxyaspergillic acid and aspergillic acid in *Aspergillus flavus* [14], or 2-methoxy-3-isopropylpyrazine in *Pseudomonas perolens* [8].

In this paper, we report on the biosynthesis of novel pyrazine metabolites by the polymyxin-producing bacterium *Paenibacillus polymyxa*. Related *Bacillus* species have previously been demonstrated to synthesise different alkyl pyrazines [3,11,12], and our results indicate the formation of a rather complex pyrazine mixture.

2. Materials and methods

2.1. Bacterial material

The *P. polymyxa* strain was obtained from the American Type Culture Collection (ATCC 10401) and routinely cultivated in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) medium (50 ml in a 250-ml Erlenmeyer flask, 30°C, 150 rpm, 52 h) for pyrazine metabolite production or in TSB medium supplemented with valine (1 g l^{-1}) (200 ml in a 1000-ml Erlenmeyer flasks, 30°C, 150 rpm, 30 h) to achieve optimal production of the 2,5-diisopropylpyrazine metabolite.

2.2. Recovery of 2,5-diisopropylpyrazine

The pyrazine metabolite subjected to ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) analysis was isolated by a purge and trap method [15]. Briefly, approximately 600 ml fermentation broth was pooled in a 1000-ml washing bottle connected to an acid trap device. The washing bottle was placed in a water bath kept at 50°C and purged with N_2 onto a 20 ml solution of 6 M HCl for 120 min. The HCl extract was carefully neutralised with NaOH and extracted with $CDCl_3$. The $CDCl_3$ extract was used for ^{1}H- and ^{13}C-NMR analysis. Purity of the extract as judged by gas chromatography-mass spectrometry (GC/MS) analysis was approximately 98%.

2.3. Isolation and identification of pyrazine metabolites

The biosynthesised pyrazine metabolites subjected to GC/MS analysis were isolated from a 20-ml sample that was collected from the bacterial suspension after 52 h of growth and transferred to a 100-ml washing bottle. Stainless steel tubes, packed with 200 mg Tenax TA® mesh 60–80 (Supelco, Pennsylvania, USA), were fixed onto the glass tubes of the insert of the washing bottle. The flask was placed in a water bath kept at 50°C and the volatile pyrazine metabolites were purged onto the trap with nitrogen at a flow rate of 100 ml min^{-1} for 30 min. The trapped volatile metabolites were thermally desorbed from the trap in a Perkin Elmer ATD400 thermal desorber (250°C, 7 min) and trapped by a cold trap (−30°C) packed with 20 mg Tenax TA® mesh 60–80. The volatiles were injected into the gas chromatograph by flash heating the trap to 300°C for 1 min with a split flow of 1:20. Transfer line temperature was 200°C. The identification of the pyrazine metabolites was based on co-elution with authentic material and comparison of electron impact ionisation (EI) mass spectra, or tentatively by comparison of the recorded mass spectra with mass spectral data from the NIST-98 database or by interpretation of the fragmentation pattern of the analysed compound.

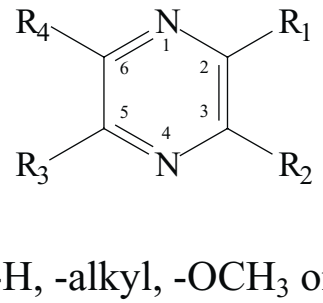

Fig. 1. General structure of a pyrazine molecule.

2.4. Analytical methods

Membrane inlet mass spectrometry (MIMS) [16] was performed using a quadrupole mass spectrometer (QMG 420, Balzers, Liechtenstein) with 70 eV ionisation energy. The membrane inlet was incorporated in a stirred sample cell (2 ml volume) as described elsewhere [17] and utilised a 125 μm thick silicone membrane (Sil-Tec Sheeting, Technical Products, Georgia, USA) as the only separation between the liquid sample and the ion source of the mass spectrometer. The microbial broth was analysed as previously described [18]. In brief, a 1-ml sample of the culture supernatant was removed from the growing culture and directly transferred to the measuring cell. After approximately 2.5 min, a steady-state flow of volatiles through the membrane and into the mass spectrometer was achieved and mass spectra were recorded.

GC/MS analysis was performed with a Hewlett-Packard 5890 series II apparatus with a 5971 mass-selective detector. Compounds were separated using a DP 5 SIL low bleed MS capillary column (60 m × 0.25 mm [inside diameter]; 1 μm film thickness) (Chrompack, USA) and helium as the carrier gas. The column was kept at 100°C for 2 min and then programmed to 280°C at 3°C min^{-1} where it was kept for 2 min. The column pressure was 90 kPa with a He flow of approximately 1 ml min^{-1}. The mass spectrometer was operated at 70 eV, scan time 0.47 s, scan range 20–350 amu, solvent delay 7 min, interface temperature 280°C and ion source temperature 180°C.

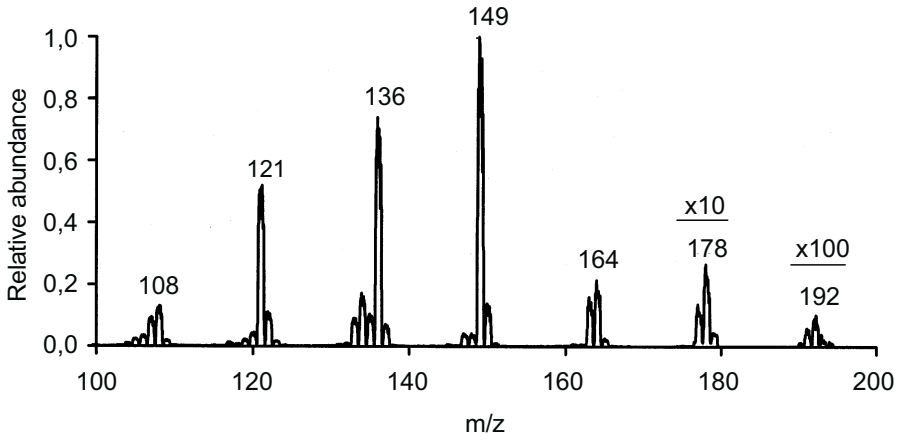

Fig. 2. MIMS spectrum of volatile metabolites in the culture fluid of *P. polymyxa* ATCC 10401 incubated for 52 h in TSB medium.

High-resolution mass spectrometry (HRMS) and MS/MS analysis were done with a Q-TOF 2® (Micromass, Manchester, UK) equipped with a Micromass MS-Nose® gas-phase inlet and run in APCI positive ion mode. Instrument settings for HRMS analysis were the following: cone voltage 13 V, mass range 50–300 Da, APCI probe temperature 130°C and source temperature 120°C, corona needle current 4.0 μA. MS/MS analysis was performed using argon as collision gas. A sealed headspace vial with 10 ml sample was thermostated to 60°C. After equilibration the seal of the headspace vial was perforated and the gas-phase sample was transferred into the mass spectrometer and accurate mass measurements of the pseudomolecular ion $(M+H)^+$ of the unknown metabolites were done.

^{1}H-NMR spectra (300 MHz, 64 scans) and ^{13}C-NMR spectra (75 MHz, 8000 scans) were recorded on a Varian Gemini-2000 spectrometer at 25°C. The solvent used was $CDCl_3$.

2.5. Chemicals

2,5-Diisopropylpyrazine and 2,6-diisopropylpyrazine were kindly provided by Dr Robert J. Bartolt, USDA Agricultural Research Service, Illinois, USA. 2-Isobutylpyrazine and 2-isopropylpyrazine was obtained from Yama Products, Utrecht, The Netherlands. All other chemicals used were of standard analytical grade purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

3. Results

3.1. Identification of 2,5-diisopropylpyrazine

Initially, we investigated the possibility of using MIMS for the monitoring of volatile metabolites produced by

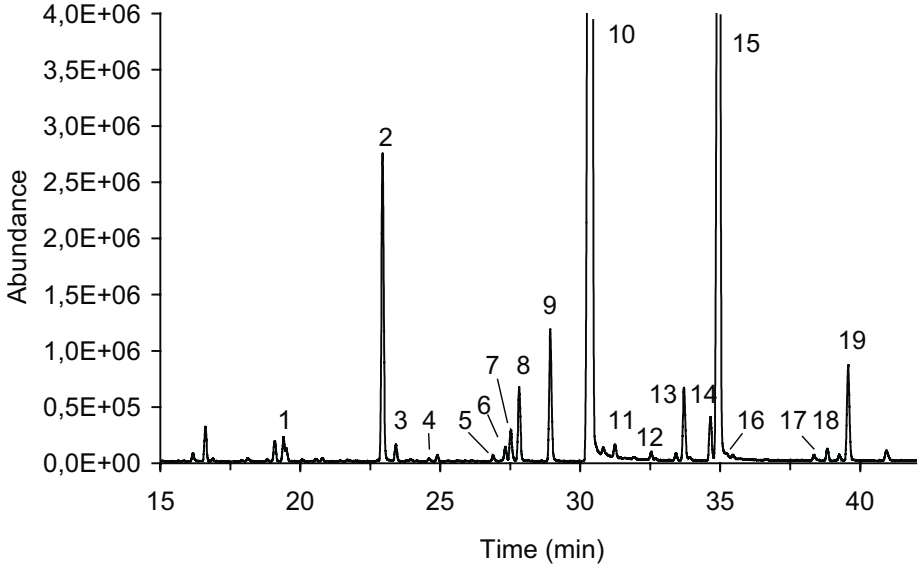

Fig. 3. Total ion chromatogram of the dynamic headspace analysis of the culture fluid of *P. polymyxa* ATCC 10401 incubated for 52 h in TSB medium.

Table 1
HRMS data of the pseudomolecular ions $(M+H)^+$, and predicted molecular formula for the observed masses of unknown volatile metabolites produced by *P. polymyxa* ATCC 10401

Observed mass (Da)	Error (ppm)	Calculated mass (Da)	Formula
123.0917	4.3	123.0922	$C_7H_{11}N_2$
137.1080	0.7	137.1079	$C_8H_{13}N_2$
151.1232	2.3	151.1235	$C_9H_{15}N_2$
165.1389	1.5	165.1392	$C_{10}H_{17}N_2$
179.1548	0.0	179.1548	$C_{11}H_{19}N_2$

P. polymyxa. With MIMS the growth medium can be analysed directly and without any pretreatment at all. However, all volatile, hydrophobic compounds are introduced into the mass spectrometer simultaneously and a complex electron impact mass spectrum may result. This was the case in our polymyxin fermentation. Fig. 2 shows the membrane inlet mass spectrum of a sample collected from the fermentation broth of *P. polymyxa* incubated for 52 h. Abundant ions at *m/z* 121, 136, 149, and 164 and ions with low intensity at *m/z* 178 and 192 indicate a mixture of metabolites that needs to be identified before the MIMS technique can be used for monitoring of the metabolites during growth.

Identification was carried out using GC/MS. Fig. 3 shows the total ion chromatogram of a 52 h old fermentation. The abundant ions at *m/z* 164 ($M^{+\bullet}$), 149 (base peak) and 136 in the MIMS spectrum (Fig. 2) were primarily due to compound **10** (retention time 30.25 min) in the gas chromatogram. The mass spectrum (Fig. 4, Table 1) of this metabolite was very similar to the spectrum observed with MIMS and accounts for most of the observed ions at masses below 164 Da. A mass spectral library search was unsuccessful. However, we found that the characteristics of the mass spectrum of **10** were similar to the mass spectrum of 2-methyl-5-isopropylpyrazine, although shifted 28 Da upwards. To gain information on the molecular composition of the unknown metabolite HRMS data were acquired (Table 1). An observed mass of 165.1389 Da for the pseudomolecular ion (MH^+) of **10** corresponded very well to the calculated mass for C_{10}-$H_{17}N_2$ (165.1392 Da) indicative of a heteroaromatic ring with two nitrogen atoms. MS/MS analysis of the ion gave fragment ions at *m/z* 150.1152, 137.0910 and 123.0919 indicative of losses of CH_3, C_2H_4 and C_3H_6. The mass spectral data suggested that the unknown metabolite was an alkyl-substituted pyrazine.

Purification of **10** was achieved by supplementing the growth medium of *P. polymyxa* with valine, a known precursor of 2-methoxy-3-isopropylpyrazine [8]. Valine supplementation favoured the production of **10** and inhibited the formation of other pyrazine metabolites. From this medium we could purify **10** to a sufficient degree for ^{1}H- and ^{13}C-NMR analysis using an acid trap purification method [15]. Resonance assignments (ppm) and coupling constants (Hz) for the metabolite were as follows: ^{1}H-NMR: δ 8.505 (s, 2H), 3.212 (sept., $J = 6.6$, 2H), 1.374 d, $J = 6.6$, 12H); ^{13}C-NMR: δ 22.083 (CH_3), 29.689 (alkyl CH), 140.904 (ring CH, heteroaromate), 159.192 (ring C). From the ^{1}H-NMR analysis the septet and duplet at δ 3.212 and 1.374 ppm were indicative of an isopropyl group and the singlet at 8.505 ppm was indicative of protons on a pyrazine ring, which in all is supported by the ^{13}C-NMR data recorded.

Combining the gathered information on molecular formula (HRMS) and structural information from MS, MS/MS, ^{1}H- and ^{13}C-NMR analysis we identified the metabolite as 2,5-diisopropylpyrazine. This identification was confirmed by co-elution of authentic material and the unknown metabolite.

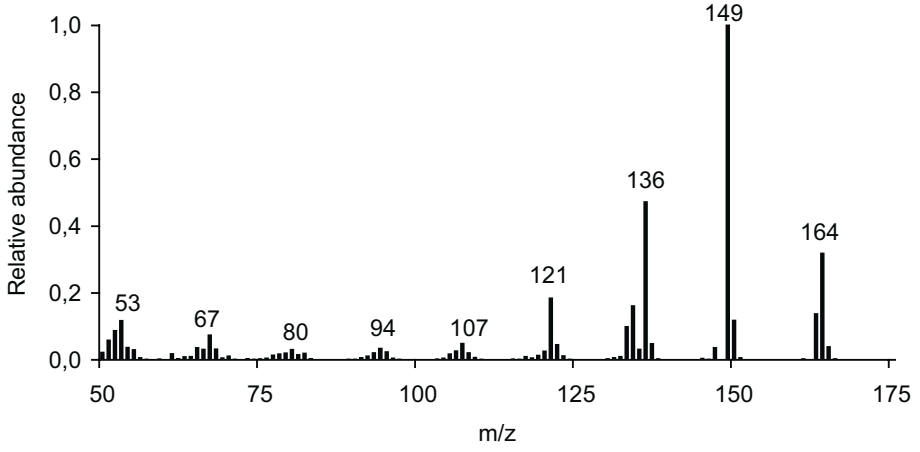

Fig. 4. EI-MS spectrum of the unknown compound **10**.

3.2. Identification of other isopropyl, isobutyl- and sec-butyl-substituted pyrazines

In addition to the metabolite identified as 2,5-diisopropylpyrazine a rather complex mixture of dialkyl- (mixtures of isobutyl and sec-butyl moieties) and methyl-dialkyl-substituted pyrazines was produced by *P. polymyxa* as identified by their characteristic fragmentation patterns in EI-MS and HRMS determination of structural formulas (Table 2 and Table 1). The methyl-branched alkyl-substituted pyrazines showed distinct fragmentation patterns highly dependent on the length of the alkyl-branched substituent and also on the position of the branch point. Common characteristic ion clusters in EI-MS spectra of alkyl-substituted pyrazines were observed at *m/z* 94 ($C_5N_2H_6^+$), 80 ($C_4N_2H_4^+$), 67 ($C_3N_2H_3^+$) and 53 ($C_3NH_3^+$). Despite their weak intensity they were diagnostic ion clusters for the identification of pyrazines in general. All pyrazine metabolites identified together with their mass spectral data are summarised in Table 2.

3.2.1. Isopropylpyrazines

Characteristic for the isopropyl-substituted pyrazines is a base peak at *m/z* (M-15) produced by methyl loss, and a pronounced (M-28) ion produced by a rearrangement reaction with concomitant loss of a $\cdot C_2H_4$ fragment. In addition to **10** eight compounds with fragmentation patterns typical for isopropyl-substituted pyrazines were identified. Compound 1 ($M^{+\bullet}$ at *m/z* 122) was identified as 2-isopropylpyrazine and compounds **2** and **3** ($M^{+\bullet}$ at *m/z* 136) as 2-methyl-6- and 2-methyl-5-isopropylpyrazine. Three compounds with $M^{+\bullet}$ at *m/z* 150 also exhibited the typical fragmentation pattern of isopropyl-substituted pyrazines. They were identified as 2,3-dimethyl-5-isopropylpyrazine (**5**), 2,5-dimethyl-3-isopropylpyrazine (**6**) and 2,6-dimethyl-3-isopropylpyrazine (**8**), respectively.

Another compound was detected (**9**, $M^{+\bullet}$ at *m/z* 164) exhibiting virtually a similar fragmentation pattern as 2,5-diisopropylpyrazine with the molecular ion at *m/z* 164 except for minor differences in the abundance of the ions at *m/z* 107 and 108 and also at *m/z* 121 and 122. Based on co-elution with authentic material and identical fragmentation pattern in EI-MS analyses this metabolite was identified as 2,6-diisopropylpyrazine. The minor peak (**16**) at retention time 35.40 min had a molecular ion at *m/z* 192 and was tentatively identified as 2,5-dimethyl-3,6-diisopropylpyrazine.

3.2.2. Isobutylpyrazines

Characteristic for the fragmentation of isobutyl-substituted pyrazines in EI-MS is a pronounced ion due to methyl loss (M-15) and base peak at *m/z* (M-42) produced by a rearrangement reaction with concomitant loss of a $\cdot C_3H_6$ fragment. Six isobutyl-substituted pyrazines were identified as metabolites of *P. polymyxa*. Isobutylpyrazine (**4**) was identified by comparison with authentic material. Based on fragmentation pattern and comparison with a mass spectral database, metabolite **7**, with a molecular ion at *m/z* 150, was identified as 2-methyl-5-isobutylpyrazine. Two metabolites with similar EI fragmentation patterns although shifted 14 Da upwards in mass range (MW 164 Da) were tentatively identified as dimethyl-isobutyl-substituted pyrazines with 2,6-dimethyl-5-isobutylpyrazine (**12**) and 2,5-dimethyl-6-isobutylpyrazine (**11**) being the most likely candidates. Two metabolites with a molecular mass of 192 Da (compounds **19** and **17**) also having a

Table 2
EI-MS and identity of pyrazines biosynthesised by *P. polymyxa* ATCC 10401 when cultivated in TSB medium

Metabolite	Retention time (min)	Identification	*m/z* of fragment ions (% relative intensity)
1	19.40	122 (M^+, 22), 107 (100), 94 (33), 80 (13), 53 (22)	2-Isopropylpyrazine
2	22.91	136 (M^+, 35), 121 (100), 108 (44), 94 (9), 66 (11), 53 (12)	2-Methyl-6-isopropylpyrazine
3	23.38	136 (M^+ 38), 135 (21), 121 (100), 108 (40), 94 (8), 66 (6), 53 (10)	2-Methyl-5-isopropylpyrazine
4	23.92	136 (M^+ 12), 121 (20), 108 (4), 94 (100), 67 (7), 52 (4)	2-Isobutylpyrazine
5	26.85	150 (M^+, 38), 149 (25), 135 (100), 122 (55), 108 (9), 80 (3), 67 (12), 53 (21)	2,3-Dimethyl-5-isopropylpyrazine
6	27.29	150 (M^+, 38), 149 (25), 135 (100), 122 (56), 108 (9), 80 (3), 67 (12), 53 (21)	2,5-Dimethyl-3-isopropylpyrazine
7	27.49	150 (M^+, 10), 135 (15), 122 (1), 108 (100), 94 (1), 80 (2), 66 (8), 53 (2)	2-Methyl-5-isobutylpyrazine
8	27.90	150 (M^+, 33), 149 (18), 135 (100), 122 (35), 107 (11), 94 (2), 80 (4), 67 (6), 53 (13)	2,6-Dimethyl-5-isopropyl-pyrazine
9	28.90	164 (M^+, 31), 163 (22), 149 (100), 136 (58), 122 (6), 107(2), 94 (2), 80 (2), 67 (6), 53 (13)	2,6-Diisopropylpyrazine
10	30.25	164 (M^+, 32), 163 (14), 149 (100), 136 (47), 121 (18), 107 (5), 94 (3), 80 (3), 67 (7), 53 (12)	2,5-Diisopropylpyrazine
11	31.88	164 (M^+ 15), 149 (25), 136 (7), 122 (100), 107 (3), 94 (2), 80 (9), 67 (3), 53 (7)	2,5-Dimethyl-6-isobutylpyrazine
12	32.50	164 (M^+ 24), 149 (36), 136 (9), 122 (100), 107 (49), 94 (4), 80 (2), 67 (5), 53 (8)	2,6-Dimethyl-5-isobutylpyrazine
13	33.67	178 (M^+ 9), 163 (16), 147 (3), 136 (100), 121 (11), 108 (2), 94 (3), 80 (1), 67 (3), 53 (4)	2-Isopropyl-6-isobutylpyrazine
14	34.61	178 (M^+ 18), 163 (47), 150 (100) 135 (57), 121 (11), 107 (3), 94 (3), 80 (4), 67 (6), 53 (14)	2-Isopropyl-5-sec-butylpyrazine
15	34.91	178 (M^+ 18), 163 (23), 150 (3), 136 (100), 121 (74), 107 (4), 94 (3), 80 (2), 67 (4), 53 (7)	2-Isopropyl-5-isobutylpyrazine
16	35.40	192 (M^+ 60), 177 (100), 164 (58), 149 (13), 135 (3), 107 (17), 94 (1), 79 (7), 67 (6), 53 (2)	2,5-Dimethyl-3,6-diisopropylpyrazine
17	38.30	192 (M^+ 10), 177 (16), 150 (100), 135 (4), 108 (30), 94 (2), 80 (3), 66 (6), 53 (3)	2,6-Diisobutylpyrazine
18	39.20	192 (M^+ 15), 177 (21), 164 (28), 150 (71), 136 (8), 121 (100), 107 (4), 94 (2), 79 (1), 67 (3), 53 (7)	2-Isobutyl-5-sec-butylpyrazine
19	39.55	192 (M^+ 14), 177 (14), 162 (1), 150 (87), 135 (5), 121 (1), 107 (100), 94 (2), 80 (4), 66 (3), 53 (4)	2,5-Diisobutylpyrazine

typical fragmentation pattern of isobutyl-substituted pyrazines were identified as 2,5- and 2,6-diisobutylpyrazine.

3.2.3. Sec-butylpyrazines

The typical fragmentation of sec-butyl-substituted pyrazines includes abundant ions at M-15, M-28, and M-43 due to $\cdot CH_3$, $\cdot C_2H_4$ and $\cdot C_3H_7$ loss. Two metabolites with molecular masses of 178 Da and 192 Da respectively with fragmentation patterns similar to the fragmentation of a sec-butyl-substituted pyrazine were tentatively identified as 2-isopropyl-5-sec-butylpyrazine (**14**) and 2-isobutyl-5-sec-butylpyrazine (**18**).

Isomers with similar fragmentation patterns typical for isopropyl-isobutyl-substituted pyrazines were identified as 2-isopropyl-5-isobutylpyrazine (**15**) and 2-isopropyl-6-isobutylpyrazine (**13**) ($M^{+\bullet}$ 178). Both compounds showed a pronounced ion at *m/z* 136 due to a rearrangement and concomitant loss of $\cdot C_3H_6$ typical of isobutyl-substituted pyrazines.

4. Discussion

Pyrazines are common molecules found in a variety of sources and are produced through chemical reactions or are formed via the primary and secondary metabolism in plants and microorganisms. Many pyrazines, e.g. 2-isopropyl-3-methoxypyrazine, have distinct sensory properties and serve as important flavour molecules in many foods. Several pyrazines are metabolites of *Paenibacillus* spp. [3,11,12] and other microorganisms [7–10]. Here we report the identification of 19 pyrazine metabolites isolated from *P. polymyxa* ATCC 10401. Twelve of the identified metabolites have to our knowledge not previously been isolated from any microbial source, and of these, seven are new natural compounds.

The most abundant pyrazine metabolite produced by *P. polymyxa* was identified as 2,5-diisopropylpyrazine. Biosynthesis of this metabolite was correlated with growth. This compound has previously been identified in oranges fed upon by the pineapple beetle *Carpophilus humeralis* [19] together with its 2,6-diisopropyl isomer. Despite the close structural similarity of these two diisopropyl-substituted pyrazines, the 2,5-isopropyl isomer is highly attractive to *C. humeralis* when used synergistically with 4-ethyl-2-methoxyphenol, 2-phenylethanol or propyl acetate whereas 2,6-diisopropylpyrazine is completely inactive [19]. Methyl- and branched alkyl-substituted pyrazines, e.g. 2,5-dimethylpyrazine and 2-methoxy-3-isopropyl-, isobutyl- or sec-butylpyrazine, have important organoleptic properties. The isobutyl-, sec-butyl-substituted pyrazine aspergillic acid is an antibiotic [5], and pyrazines produced by toxic insects serve as warning signals [20], which in all indicate versatile biological properties of pyrazine molecules. We identified seven new natural pyrazine molecules: 2-isopropyl-6-isobutylpyrazine, 2-isopropyl-5-sec-butylpyrazine, 2-isopropyl-5-isobutylpyrazine, 2,5-dimethyl-3,6-diisopropylpyrazine, 2-isobutyl-5-sec-butylpyrazine, and 2,5- and 2,6-diisobutylpyrazine. These new metabolites might have important biological activities such as new organoleptic properties, or, because of their structural similarity with aspergillic acid, antimicrobial activities.

Several authors suggest a pathway for pyrazine biosynthesis initiated by the formation of a cyclic peptide through the condensation of two amino acids such as the formation of pulcherriminic acid via the condensation of two leucine molecules to cycloleucyl-leucyl [13], or the formation of 2-isopropyl-3-methoxypyrazine from the initial condensation of the amino acids valine and glycine [8]. This pathway allows only the formation of 2,5-dialkyl-substituted metabolites. Another biosynthetic pathway for alkyl-substituted pyrazine formation is suggested by Murray et al. [1]. The initial reaction of this pathway is the condensation of an α,β-dicarbonylic compound and an amidated amino acid and allows the formation of both 2,5- and 2,6-alkyl-substituted pyrazine molecules.

The presence of both 2,5- and 2,6-dialkyl-substituted isomers such as 2,5- and 2,6-diisopropylpyrazine and 2,5- and 2,6-diisobutylpyrazine suggests that the pathway utilising a condensation reaction between an α,β-dicarbonylic compound and an amidated amino acid is active in *P. polymyxa*. We also found that supplementation of valine led to an increased production of 2,5-diisopropylpyrazine and an inhibition of the formation of all the other pyrazine metabolites. This indicates that valine is the precursor of the 2,5-diisopropyl-substituted pyrazine molecule. Our future research will focus on this issue.

In conclusion, we identified 19 alkyl-substituted pyrazine metabolites isolated from the fermentation broth of *P. polymyxa*. Twelve of these compounds are novel microbial metabolites, and of these, seven are new natural compounds.

Acknowledgements

Tabisham Khan and Jonathan Pough, Micromass Ltd, Manchester, UK are greatly acknowledged for their assistance with performing high-resolution mass spectrometry. Brian Gregersen and Paul Stein are acknowledged for the NMR analysis, and Anita Lunding and Bente Lambertsen for skilled technical assistance.

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