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D. Parrot, L. Intertaglia, P. Jéhan, M. Grube, M.T. Suzuki, et al.. Chemical analysis of the Alphaproteobacterium strain MOLA1416 associated with the marine lichen *Lichina pygmaea*. *Phytochemistry*, 2018, 145, pp.57-67. 10.1016/j.phytochem.2017.10.005 . hal-01639693

HAL Id: hal-01639693

<https://univ-rennes.hal.science/hal-01639693>

Submitted on 18 May 2020

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Chemical analysis of the Alphaproteobacterium strain MOLA1416 associated with the marine lichen *Lichina pygmaea*

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Abstract

Alphaproteobacterium strain MOLA1416, related to *Mycoplana ramosa* DSM 7292 and *Chelativorans intermedius* CC-MHSW-5 (93.6% 16S rRNA sequence identity) was isolated from the marine lichen, *Lichina pygmaea* and its chemical composition was characterized by a metabolomic network analysis using LC-MS/MS data. Twenty-five putative different compounds were revealed using a dereplication workflow based on MS/MS signatures available through GNPS (<https://gnps.ucsd.edu/>). In total, ten chemical families were highlighted including isocoumarins, macrolactones, erythrinan alkaloids, prodiginines, isoflavones, cyclohexane-diones, sterols, diketopiperazines, amino-acids and most likely glucocorticoids. Among those compounds, two known metabolites (**13** and **26**) were isolated and structurally identified and metabolite **26** showed a high cytotoxic activity against B16 melanoma cell lines with an IC₅₀ 0.6 ± 0.07 µg/mL.

Keywords: Lichen-associated bacteria; *Lichina pygmaea*; Alphaproteobacteria; LC/MSⁿ; Dereplication workflow; Molecular networking

1 Introduction

The length of time necessary for the discovery of novel bioactive compounds in complex matrices remains a challenge for natural products research. To facilitate and accelerate analysis and identification using mass spectrometry data, several computational approaches were recently developed (Allen et al., 2014; Cao et al., 2013; Dührkop et al., 2015; Heinonen et al., 2012; Hufsky et al., 2014; Vaniya and Fiehn, 2015). Among those, molecular networking analysis is a (MS/MS)-based computational approach that allows multiple comparisons and represents a rapid method of dereplication and identification of novel compounds. Molecular networking is based on comparisons to known structural scaffolds in databases using authentic standards, and can be combined with *in silico* MS/MS fragmentation databases (Allard et al., 2016; Yang et al., 2014). As an example, GNPS (Global Natural Products Social

molecular networking), is an open-source advanced data analysis platform that includes tools for dereplication, comparative dereplication, molecular networking and automated data reanalysis (<http://gnps.ucsd.edu> (Wang et al., 2016)). Here, we applied this recently-developed approach to study the chemical profiles of bacterial communities associated with a marine lichen.

Lichens are symbiotic communities of a shape-providing ascomycete that shelters extracellular photosynthesizing partners (a green alga and/or a cyanobacterium). This self-sustaining association provides an ecological niche for additional microorganisms that have been identified by culture independent gene-sequencing studies as lichenicolous fungi, as well as a plethora of non-photosynthetic bacteria (eg. Bates et al., 2011; Cardinale et al., 2008, 2006; Grube et al., 2014, 2009; Hodkinson and Lutzoni, 2010; Selbmann et al., 2009, Spribille et al., 2016). Several studies concerning bacteria were also conducted by culture-dependent approaches (recently reviewed by Suzuki et al., 2016; Aschenbrenner et al., 2016). Among bacterial metabolites retrieved from these lichen-associated communities, some showed bioactivity (Parrot et al., 2016a). Those include angucycline and butenolide (Motohashi et al., 2010), coumabiotics A-F (Cheenpracha et al., 2010), cladoniamides A-G (Williams et al., 2008), unciamycin (Davies et al., 2005) and the more recently described unciaphenol (Williams et al., 2015). Finally, we have recently isolated the two new acrylate compounds, cyaneodimycin and its derivative cyaneomycin, from isolates of *Streptomyces cyaneofuscatus* MOLA1488 isolated from *Lichina confinis* (Parrot et al., 2016b).

In the current study, we have focused on the intertidal lichen *Lichina pygmaea* from which we isolated 24 different strains (Parrot et al., 2015), and in particular, Alphaproteobacterium strain MOLA1416 likely corresponding, based on 16S rRNA gene sequence analysis (Parrot et al., 2015) to a putative new bacterial species if not a new genus in the order Rhizobiales. We selected this strain based on the cytotoxic activity of its raw organic extracts (IC₅₀ 7.2 ± 0.8 µg/mL against B16 melanoma cell lines). Furthermore, no secondary metabolite had ever been reported for the two bacterial species (*Mycoplana ramosa* and *Chelativorans intermedius*) most closely related to MOLA1416. Strain MOLA1416 was subject of a dereplication workflow and molecular networking analysis using GNPS along with seven Actinobacteria isolated from either a subtidal lichen (*L. confinis*) or a supralittoral lichen (*Rocella fuciformis*; Table 1) (Parrot et al., 2015) occurring in the same geographic region as MOLA1416. Our main goal was to study the chemical diversity of MOLA1416 by classifying its metabolites in chemical structural families and by structurally identifying some of these metabolites.

Table 1 Characteristics of bacteria isolated from *Lichina pygmaea*, *L. confinis* and *Rocella fuciformis* (Parrot et al., 2015) used in the molecular networking by GNPS (<http://gnps.ucsd.edu>).

alt-text: Table 1							
Lichens	Bacterial strains	Genus	16S rRNA similarity (%)	Family	Production of metabolites	IC ₅₀ on B16 cell lines of AcOEtEtOAc extract (µg/mL)	References
<i>Lichina pygmaea</i> (marine)	MOLA1416	<i>Mycoplana ramosa</i> ^a <i>Chelativorans intermedius</i> ^a	93.56 93.56	Brucellaceae Phyllobacteriaceae	– –	7.2 ± 0.8	(Kämpfer et al., 2015; Urakami et al., 1990)
<i>Lichina confinis</i> (marine)	MOLA1488	<i>Streptomyces cyaneofuscatus</i>	100.00	Streptomycetaceae	daunomycin, cosmomycin B, galtamycin B, maltophilin, cyaneodimycin, cyaneomycin, 3-(hydroxyacetyl) indole, <i>cyclo</i> -(Phe, Pro), <i>cyclo</i> -(L-Leu, L-Pro), <i>N</i> -acetyl-β-oxotryptamine, <i>N</i> -methylactinomycin, usnic acid	0.33 ± 0.16	(Braña et al., 2015; Parrot et al., 2016b)
	MOLA1448	<i>Marmoricola aequoreus</i> ^a	95.04	Nocardioidaceae	NF	83 ± 10	–
	MOLA1450	<i>Micrococcus luteus</i>	97.93	Micrococcaceae	lutoid, [3H]-indole acetic acid	36 ± 4	(Barazani and Friedman, 1999; Bultel-Poncé et al., 1997)
<i>Rocella fuciformis</i> (maritime)	MOLA1522	<i>Nocardioides mesophilus</i> ^a	94.91	Nocardioidaceae	teichoic acid, leucylblaticidin, rodaplutin (<i>Nocardioides</i> sp.)	250 ± 10	(Dellweg et al., 1988; Shashkov et al., 1999)
	MOLA1528	<i>Sedimihabitans luteus</i>	98.43	Cellulomonadaceae	NF	>400	–
	MOLA1554	<i>Sedimihabitans luteus</i>	98.07	Cellulomonadaceae	NF	215 ± 35	–
		<i>Microbacterium</i>					

	MOLA1545	<i>pumilum</i>	99.88	<i>Microbacteriaceae</i>	exopolysaccharides (<i>Microbacterium</i> sp.)	>400	(Matsuyama et al., 1999)
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^a Putative new species based on 16S rRNA gene sequence analysis (identity ≤ at 97%); NF: not found.

2 Results and discussion

2.1 Molecular networking and dereplication approaches

A LC-UV/visible low-resolution MS/MS approach was performed to describe the qualitative chemical diversity of strain MOLA1416. The combined of detection by UV:Vis photodiode array (PDA) and electrospray ionization mass spectrometry (ESI-MS), both coupled to a C₁₈ reversed phase HPLC, provided an accurate method for determining the chemical profile of the strain (Fig. 1). A PDA chromatogram (between 200 and 600 nm; Fig. 1A) and base peak chromatograms in both positive and negative ion modes (Fig. 1B-C) were obtained. MS parameters were optimized using the small generic and synthetic compounds NH₃⁺-Phe-Phe-OMeHCl⁺ for the positive mode and *N*-Boc-Phe-Phe-COO⁻Na⁺ for the negative mode, both dissolved in MeOH/acetonitrile (50:50) and injected onto the mass spectrometer using a syringe pump. Optimized parameters permitted the visualization of several compounds. Nevertheless, only the positive mode was considered subsequently as it allowed the detection of a larger number of compounds.

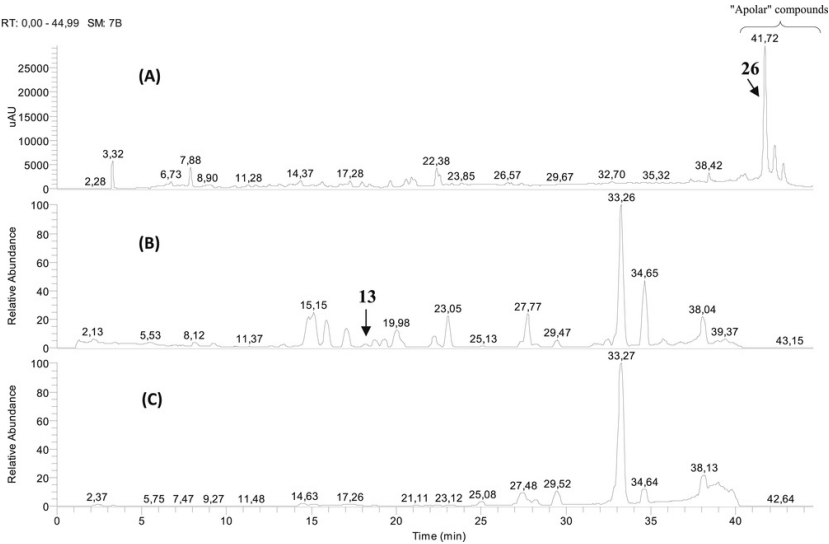


Fig. 1 PDA Chromatogram (at 280 nm) **(A)** and base peak chromatograms in positive mode **(B)** and negative mode **(C)** of MOLA1416 (**13** and **26** correspond to compounds isolated).

alt-text: Fig. 1

Mass spectrometry data were used to facilitate the chemical analysis of the MOLA1416 strain. Dereplication and molecular networking workflows were performed using GNPS based on similar fragmentation spectra. The dereplication workflow analysis revealed up to twenty-five putatively different known compounds (Table 2, Figs. 2 and 3). Among them, several chemical families including isocoumarins (**1**), isoflavones (**2**), macrolactones (**3**), erythrinan alkaloids (**4**), glucocorticoids (**5** to **7**), cyclohexane-diones (**8**) sterols (secondary metabolites; **9**) as well as amino acids (primary metabolites; **10**) and diketopiperazines (**11** to **25**) were highlighted (Table 2 and Fig. 3). Diketopiperazines are already known for their biological properties (Huang et al., 2010; Martínez-Luis et al., 2012; Martins and Carvalho, 2007; Rui et al., 2013; Soliev, 2012). Surprisingly, some metabolites revealed structural similarities with synthetic compounds [betapar (**5**), budesonide (**6**), prednisone (**7**) and trinexapac (**8**)] analogous to vertebrate steroid hormones. Sterol biosynthesis is relatively rare among bacteria but some species are know to synthesize sterol-like compounds such as hopanoids (Rohmer et al., 1984), sterols (Wei et al., 2016) and even glucocorticoid-like 3-keto sterols (Simmons et al., 2011). Interesting among alphaproteobacteria, strains in the order Rhizobiales to which MOLA 1416 belongs have been shown to produce hopanoids (*Agrobacterium tumefaciens*; Rohmer et al., 1984) and cycloartenol (*Methyloceanibacter caenitepidi*; Simmons et al., 2011). Overall our results point to several possibilities: 1) MOLA 1416 produced glucocorticoid-like compounds 2) they were misidentified due to the limitation of low resolution mass spectrometry and/or due to the absence of more relevant metabolites in spectral databases available in GNPS and 3) one cannot entirely rule out contamination arising possibly from the culture medium, but the fact that these compounds were only found in extracts of this bacterium and not others prepared and analyzed under the same conditions

somewhat weakens this possibility. Among all putative chemical families revealed, only diketopiperazines (Cho et al., 2012; Li et al., 2011) and genistein (2) (Hawas et al., 2009; Huang et al., 2013; Tchize et al., 2009; Wu et al., 2007) were previously reported as bacterial secondary metabolites.

Table 2 Identification of compounds from MOLA1416 using the dereplication workflow in GNPS (<http://gnps.ucsd.edu>) in positive mode with a cosine ≥ 0.5 ; t_R in MOLA1416 raw extract; m/z of the library compounds; $\Delta m/z$: difference between the m/z of compounds on MOLA1416 extract and m/z of library compounds and shared peaks: number of ions fragments shared between the spectrum from MOLA1416 and the library spectra.

alt-text: Table 2									
	Identification	t_R (min)	m/z	$\Delta m/z$	Cosine	Shared peaks	Chemical family	Sources	References
1	3-(3,4-Dihydroxyphenyl)-8-hydroxyisochromen-1-one	29.23	271.06	1.849	0.60	6	isocoumarin	<i>Botrichium lanuginosum</i> , <i>Hydrangea macrophylla</i> var. <i>thunbergii</i>	(Zhang et al., 2009)
2	Genistein	12.47	271.20	0.420	0.52	6	isoflavone	<i>Cytisus</i> sp.	(Pereira et al., 2012; Raja et al., 2007)
3	10,11-Dehydrocurvularin	33.71	291.13	1.905	0.54	6	fungal phytotoxin	<i>Aspergillus terreus</i>	(Xu et al., 2013)
4	8-Oxoerythraline epoxide	4.43	296.10	0.950	0.55	7	alkaloïd	<i>Erythrina verna</i>	(Callejon et al., 2014)
5	Betapar	32.17	373.16	1.924	0.61	6	glucocorticoïd	Synthesis	(Fernández et al., 2003)
6	Budesonide	32.60	431.24	0.917	0.52	6	glucocorticoïd	Synthesis	(Thalén and Brattsand, 1979)
7	Prednisone	37.99	359.19	1.946	0.54	9	glucocorticoïd	Synthesis	(Li et al., 2009)
8	Trinexapac	2.55	225.08	1.764	0.69	6	cyclohexane- dione	Synthesis	(Cooper and Storey, 2006)
9	Glycocholic acid	28.30	466.00	0.040	0.84	7	sterol	Rat hepatocytes	(Iga and Klaassen, 1982)
10	L-arginine	2.64	176.12	1.340	0.75	6	amino acid	Many sources (plants, fungi, bacteria, Ascidia, etc.)	(Diak, 1977; Shan et al., 2010; Work, 1949)
11	cyclo-(D-Trp, L-Pro)	20.74	284.14	0.059	0.91	8	diketopiperazine	Many sources (sponges, fungi, etc.)	(Cho et al., 2012; Huang et al., 2010; Kelecom, 2002)
12	cyclo-(L-Trp, L-Pro)	22.54	284.14	0.089	0.91	7			
13	cyclo-(L-Leu, L-Pro)	18.15	211.14	0.033	0.96	8			
14	cyclo-(L-Phe, D-Pro)	22.15	245.13	0.008	0.78	7			
15	cyclo-(L-Tyr, L-Pro)	13.77	261.12	0.037	0.75	7			
16	cyclo-(Phe, 4-hydroxy-Pro)	15.84	261.12	0.034	0.83	7			
17	cyclo-(Asn, Leu)	11.99	228.13	1.713	0.74	6			
18	cyclo-(L-Pro, L-Val)	15.67	197.13	0.022	0.96	7			
19	cyclo-(Leu, Leu)	25.25	227.17	0.066	0.95	6			
20	cyclo-(Leu, Phe)	25.66	261.16	0.519	0.94	8			
21	cyclo-(Val, Leu)	22.62	213.16	0.219	0.93	8			
22	cyclo-(Val, Phe)	23.94	247.14	0.103	0.94	6			

23	cyclo-(gly, L-Phe)	14.47	205.07	0.026	0.98	8
24	cyclo-[(L-4-hydroxy-Pro)-L-Leu]	16.05	227.14	0.358	0.92	8
25	cyclo-[Tyr-(4-hydroxy-Pro)]	10.61	277.12	0.047	0.93	10

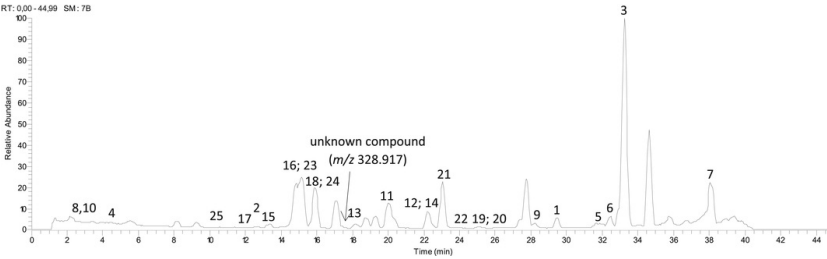


Fig. 2 Annotated mass spectrum in positive mode of MOLA1416 raw extract. Numbers correspond to compounds identified by the dereplication workflow using GNPS (Table 2).

alt-text: Fig. 2

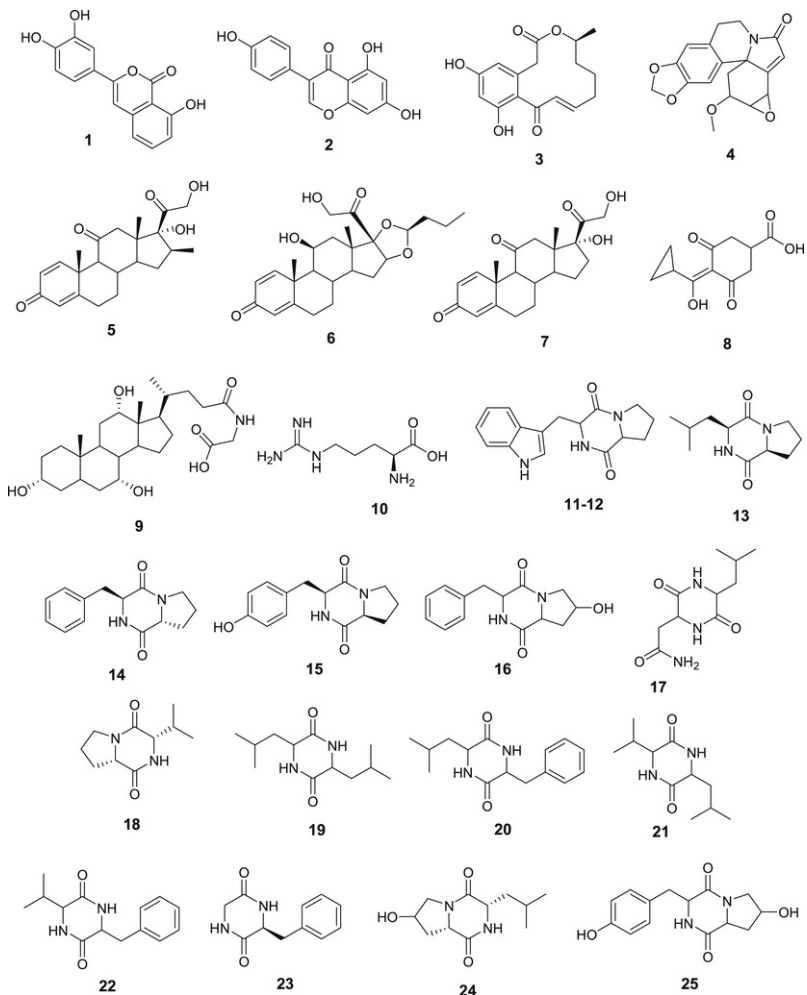


Fig. 3 Chemical structure of MOLA 1416 metabolites identified by the dereplication workflow in the positive mode using GNPS (Number relative to those of [Table 2](#)).

alt-text: Fig. 3

The results of the molecular networking analysis performed with MOLA1416 along with seven Actinobacteria strains associated with littoral lichens is shown in [Table 1](#). Among the Actinobacteria strains were members of the Streptomycetaceae (MOLA1488), Nocardioidaceae (MOLA1448 and MOLA1522), Promicromonosporaceae (MOLA1450), Microbacteriaceae (MOLA1545) and Cellulomonadaceae (MOLA1528 and MOLA1554) strains ([Table 1](#)). Among these Actinobacteria strains, three (MOLA1488, MOLA1448 and MOLA1450) associated with *L. confinis* show bioactivity (IC_{50} of raw extract against B16 melanoma cell lines below 100 $\mu\text{g/mL}$, [Table 1](#)). Among the four remaining strains associated with *R. fuciformis*, raw extracts of two (MOLA1522 and MOLA1554) showed a cytotoxic activity against B16 melanoma cell lines below 300 $\mu\text{g/mL}$ ([Table 1](#)). Metabolites that were already isolated from these selected strains are listed in [Table 1](#).

GNPS allowed clustering of MS/MS data and to compare the chemical diversity of MOLA1416 relative to the seven Actinobacteria strains ([Fig. 4](#)). The results revealed the presence of several clusters (with a mass range between m/z $[M+H]^+$ 131 and m/z $[M+H]^+$ 1295) where metabolites of MOLA1416 were also observed in the other bacterial strains. Among these clusters, three contained known compounds and consequently indicated the presence of several putative derivatives ([Figs. 4 and 5](#)). As an example, diketopiperazines, relative close chemical structures, or compounds possessing similar fragmentation patterns were associated to Cluster A and B ([Fig. 5](#)). The frequent

retrieval of diketopiperazines in all eight bacterial strains studied confirmed an absence of specificity of this chemical family (Fig. 5) which is already known to be widely distributed in fungi, bacteria, bacteria associated with sponges and many others (e.g. Cho et al., 2012; Huang et al., 2010; Kelecom, 2002). Moreover, diketopiperazines belong to a family of signaling metabolites and play roles in regulating different bacterial functions that are widespread, such as antibiotic biosynthesis, production of virulence factors, exopolysaccharide biosynthesis and bacterial swarming among others (Brelles-Mario and Bedmar, 2001).

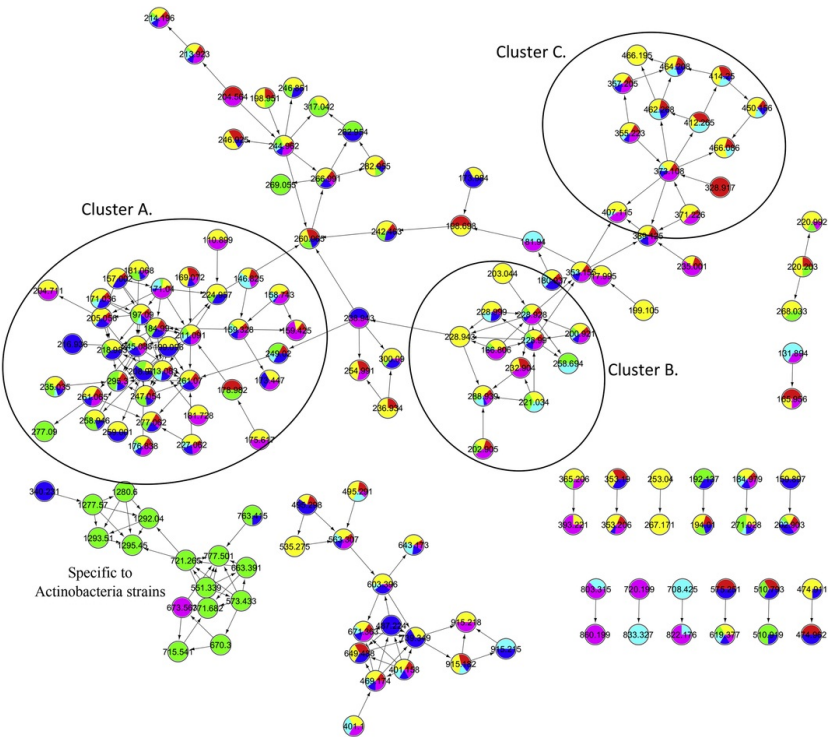


Fig. 4 Molecular networking of MS spectra in positive mode using Cytoscape® visualization highlighting three clusters (A-C) containing known compounds (details in Fig. 5) from MOLA1416 and seven other cultivable bacteria associated with marine/maritime lichens (red: *Alphaproteobacterium* (MOLA1416); green: *Streptomyces* sp. (MOLA1488); magenta: *Cellulomonadaceae* (MOLA1554, MOLA1528); blue: *Promicromonosporaceae* (MOLA1450); yellow: *Nocardiaceae* (MOLA1448, MOLA1522); cyan: *Micrococcaceae* (MOLA1545)). The coloring of each node is a pie, and the proportion colored of that pie is the proportion of the spectral counts coming from each respective group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alt-text: Fig. 4

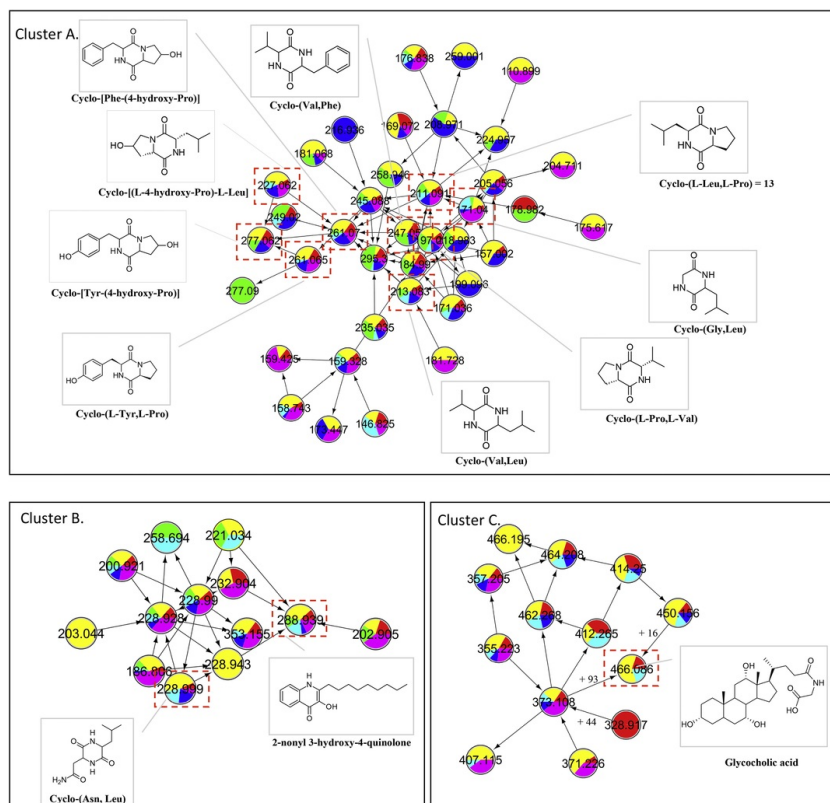


Fig. 5 Molecular networking of MS spectra in positive of cluster A to C visualized by Cytoscape® including known compounds. MOLA1416 and seven other cultivable bacteria associated with marine/maritime lichens (red: *Alphaproteobacterium* (MOLA1416); green: *Streptomyces* sp. (MOLA1488); magenta: *Cellulomonadaceae* (MOLA1554, MOLA1528); blue: *Promicromonosporaceae* (MOLA1450); yellow: *Nocardiaceae* (MOLA1448, MOLA1522); cyan: *Micrococcaceae* (MOLA1545). The coloring of each node is a pie, and the proportion colored of that pie is the proportion of the spectral counts coming from each respective group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alt-text: Fig. 5

The third cluster (Cluster C) seems to be related to compounds with fragmentation spectra similar to glycocholic acid and by extension to sterols (Fig. 5). Interestingly, a compound specific to MOLA1416 (red node in Fig. 5) was identified in cluster C. This unknown metabolite with a m/z $[M+H]^+$ 328.917 (t_R 17.48 min) could be structurally similar to glycocholic acid (9) or could have yielded a similar fragmentation pattern. It presents a mass difference of 44 (potentially corresponding to the loss of a CO_2 group) to another unidentified compound (m/z $[M+H]^+$ 373.108) itself correlated to glycocholic acid with a mass difference of 93 (potentially corresponding to a loss of C_6H_5O or C_6H_7N group) (Fig. 5). However, without any additional information, we hypothesize this compound from MOLA1416 belongs to a family putatively identified as sterols.

In addition to these three clusters, some other clusters were identified but, without known representative metabolites, no chemical family has been proposed for them. Overall, these results demonstrate the possible ability of strain MOLA1416 either to produce these metabolites from these families, or to produce metabolites with a close structural similarity and/or other compounds with very similar fragmentation patterns. Overall the results show that metabolomic approaches using molecular networks based on MS/MS similarities can not only identify known compounds, but also cluster similar chemical compounds and putatively identify the production of compounds in specific chemical families accelerating dereplication of molecules produced by different biological sources.

Following dereplication by molecular networking, we focused on “unknown” compounds, in an attempt to characterize and discover putative new, bioactive and specific metabolites associated with MOLA1416. Unfortunately, the compound specific to MOLA1416 (m/z $[M+H]^+$ 328.917) found in cluster C was one of its minor metabolites and could not be isolated in sufficient amount for structural characterization. To broaden the chemical characterization of the strain we then focused on the more “apolar” compounds identified in the PDA chromatogram (Fig. 1A) and that were not ionized under our analytical conditions.

2.2 Isolation process and chemical identification

The EtOAc organic extract (360 mg) was subjected to flash chromatography (CH₂Cl₂/EtOAc/MeOH gradient) and was partitioned using semi-preparative silica TLC leading to the isolation of two compounds: one “polar” (**13**) [using CH₂Cl₂/EtOAc/MeOH (15/15/3; v/v/v)] and one “apolar” (**26**) [using CH₂Cl₂/EtOAc (95/5; v/v)] (Fig. 6). Those metabolites, *Cyclo*-(L-Leu, L-Pro) (**13**) (2.79 mg; *t_R* 18.15 min) (Begum et al., 2014; Bernart and Gerwick, 1990; Martínez-Luis et al., 2012; Rui et al., 2013; Yang and Cordell, 1997) and 6-methoxy-2-methyl-3-methylheptylprodiginin (**26**) (25 mg; *t_R* 41.72 min) (Miao et al., 2013; Soliev, 2012) had been already isolated from several biological sources such as bacteria, sponges and fungi among others. Compound **13**, also highlighted during the GNPS dereplication (Table 2) and molecular networking with a *t_R* at 18.15 min in the raw extract confirms the prediction by the GNPS dereplication workflow analysis, and increases information of a chemical family of metabolites present in complex biological samples. Moreover, a synthetic *cyclo*-(L-Leu, L-Pro) was also analyzed by LC-MS/MS analysis with a *t_R* 18.15 min. This last information inferred the *L,L* configuration of compound **13**. Structural characteristics (NMR data) for these two compounds (**13** and **26**) are available as supplementary data files (Figs. S2–S3).

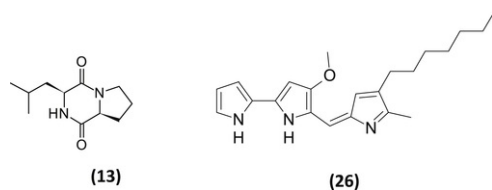


Fig. 6 Chemical structure of compounds isolated (**13** and **26**): **13**. Cyclo-(L-Leu,L-Pro) and **26**. 6-methoxy-2-methyl-3-methylheptylprodiginin.

alt-text: Fig. 6

Metabolite (**26**) had previously been reported to show cytotoxic activities against L5178Y mouse lymphocytes (IC₅₀ 0.677 μM) (Soliev, 2012) and against S-Jurkat T cells (IC₅₀ 2.27 μM) (Ranches et al., 2013). In the present study, cytotoxic activity of compound **26** was shown against HaCaT (IC₅₀ = 2.6. 10⁻² ± 1.3. 10⁻² μg/mL) and B16 (IC₅₀ = 0.6 ± 0.07 μg/mL) cell lines confirming its biological interest. This metabolite, which constituted 7% of the total mass of an unfractionated sample, was sufficient to account for the observed cytotoxicity of the crude extract. This metabolite was the main constituent responsible for the cytotoxicity of the raw extract since it represented 7% of the total mass of the crude extract.

3 Conclusion

A chemical profiling of the Alphaproteobacterium strain MOLA1416 was performed by LC/MSⁿ after optimization of ionization parameters. A dereplication workflow and molecular networking analysis using GNPS, and structural analysis led to 1) the identification of two known compounds (**26** and **13**) and 2) the putative identification of twenty-five known compounds (**1** to **25**) or compounds with relative close chemical structures and/or chemical compounds with similar fragmentation patterns. In addition, we have shown that a combination of these different approaches facilitated the analysis of mass spectrometry data, decreased the time of analysis and gave a more global vision of the chemical diversity associated with the organisms, prior to more time-consuming procedures for separation and structural identification. Furthermore, due to the presence of a scalable database on the GNPS platform, analysis and chemical identification of metabolites could be repeated and chemical profiles easily updated. Further, the approach presented here enabled efficient analysis of the biosynthetic potential of bacterial strains associated to lichens. Lichens host a large number of different bacteria (Aschenbrenner et al., 2016), but only a few of these have been characterized. These few strains already show a promising capacity for production of specialized bioactive compounds. We argue that lichens are a treasure chest for bacterial potential which has to be exploited. In the future, we plan to extend these studies to lichens from another environments.

4 Experimental

4.1 Chemicals and reagents

All commercial reagents were purchased from Carlo Erba Reactifs and/or from Sigma Aldrich (Val de Reuil, France and St Quentin Fallavier, France). For chromatographic analysis, HPLC and LC/MS grade water was obtained using an EasyPure (Barnstead, USA) water purification system. Deuterated solvents were purchased from Euriso-top (Gif-sur-Yvette, France).

4.2 Microorganism

MOLA1416 was isolated from the lichen *Lichina pygmaea* collected in Erquy (Northwest of Rennes, France, 48°37'45" N, 02°28'30" W) in April 2012 (Parrot et al., 2015). To identify the strain, its 16S rRNA gene was sequenced using the dideoxy termination Sanger sequencing as previously described (Parrot et al., 2015). Comparison with sequences in the EZtaxon server type strain database (Kim et al., 2012) revealed that the closest phylogenetic neighbor of the strain was *Mycoplana ramosa* DSM 7292 or *Chelativorans intermedius* CC-MHSW-5, both with 93.6% sequence identity. The strain was cryopreserved at -80 °C in 35% v/v glycerol or 5% v/v DMSO after growth in marine broth medium (MB) (Difco®) (Banyuls/Mer collection, reference: MOLA1416).

4.3 Culture, extraction, isolation and purification procedure

4.3.1 Fermentation of strain MOLA1416

Strain MOLA1416 was cultivated in 50 mL test tubes containing 30 mL of Marine Broth (MB, Difco) medium. Test tubes were shaken on an orbital shaker (110 rpm) at 25 °C for 72 h. Nine flasks of 300 mL of liquid MB medium were each inoculated with 3 mL of the test tubes cultures. Flasks were incubated for 14 days at 25 °C with 110 rpm shaking. Prior to harvesting of cultures, 5 g of sterile resin XAD7HP (Sigma-Aldrich, St Louis, Missouri), were added to each flask and incubated under growth conditions (25 °C, 110 rpm) for 4 h. Cultures were centrifuged at 5000 rpm for 15 min at 4 °C. Supernatants were removed and solid residues (resin and bacteria cells) were lyophilized before further experimentation.

4.3.2 Production of raw extracts of strain MOLA1416

The solid residue of the combined 2.7 L fermentation cultures was extracted at least three times with acetone/MeOH (50/50, v/v) successively. Acetone/MeOH extracts were dried under vacuum and extracted at least three times with EtOAc/H₂O (3/1, v/v). The organic phase (EtOAc extract) was collected and filtered through a 0.45 µm Nylon filter membrane (F54N45, AIT, France) Residual H₂O was removed by the addition of anhydrous sodium sulfate power (5 g) and the organic phase was dried under vacuum yielding ca. 360 mg of crude extract. The crude extract was used for LC-MS/MS analysis and was also partitioned using various preparative methods (SPOT Flash liquid chromatography (Armen Instrument®) and preparative TLC.

4.3.2.1 Isolation using C-18 flash chromatography C-18 flash chromatography was performed using a SPOT®Flash liquid chromatography (Armen Instruments). The stationary phase consisted of a silica (SiO₂) column pre-packed normal phase (FSHP-1207-0025, 25 g, Biotage) and the mobile phase a gradient: CH₂Cl₂(A)/EtOAc (B) fractions (100:0 to 0:100 in 75 min). The following gradient was applied at a flow rate of 10 mL/min: initial, 100% B; 0-10 min, 100% B linear; 10-40 min 50% B linear; 40-45 min 50% B linear; 45-75 min, 0% B linear followed by washing the column with 100% MeOH for 30 min. 105 fractions (f1-f105) of 10 mL each were collected.

4.3.2.2 Preparative TLC After a first separation by flash chromatography (cf. 4.3.4.1), two pools, F1 (f12-f38; 44 mg) and F2 (f55-f59; 6 mg) were purified by preparative TLC (10-12 µm, Kieselgel 60 F₂₅₄, Merck 5554) using a model III automatic TLC (Camag®). Two systems solvents were applied: CH₂Cl₂/EtOAc (95/5, v/v) for F1 and CH₂Cl₂/EtOAc/MeOH (15/15/3, v/v/v) for F2 and led to the isolation of two compounds (**13** and **26**).

4.3.3 Fermentation and extraction in small scale (other strains)

Other bacterial strains used were cultivated in 50 mL test tubes containing either 30 mL of **Luria-Bertani (LB) broth** fermentation broth: [5 g yeast extract (Sigma-Aldrich, St Louis, Missouri), 10 g malt extract (Sigma-Aldrich, St Louis, Missouri) and 5 g NaCl (Sigma-Aldrich, St Louis, Missouri) in 1L for MOLA1488] or Marine Broth (MB) for MOLA1450, MOLA1522, MOLA1528, MOLA1545, MOLA1554 and MOLA1528. Tubes were incubated at 25 °C for 14 days with 110 rpm shaking. The same extraction procedure was applied (cf. 4.3.2) and EtOAc extracts were obtained for all seven strains. These crude EtOAc extracts were used for LC-MS/MS analysis.

4.4 LC-ESI-MS/MS analysis

Mass spectrometry analyses were carried out on a LC-ESI and ESI-MSⁿ mass spectrometer as already described (Parrot et al., 2013). A Prevail C18 column (5 µ, 250 × 4.6 mm, GRACE®) kept at 30 °C was used. For HPLC a gradient system was applied: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The following gradient was applied at a flow rate of 1 mL/min in the HPLC system: initial, 99% A; 0.01-5 min, 90% A linear; 5-7 min, 90% A linear; 7-15 min 75% A linear; 15-17 min 75% A linear; 17-25 min 50% A linear; 25-27 min 50% A linear; 27-40 min 0% A linear; followed by washing and reconditioning of the column. A split to 0.2 mL/min was applied before the mass spectrometry system and 20 µg were injected. The MSⁿ spectra were recorded during the HPLC run using the following conditions: MS/MS analysis with starting collision induced dissociation energy of 35 eV and data analyses were performed using Xcalibur v1.0.

4.4.1 Dereplication workflow and molecular networking

All MS data were converted to mzXML format using msconvert, a part of the ProteoWizard package (Chambers et al., 2012) and imported into the GNPS platform (<http://gnps.ucsd.edu>, Wang et al., 2016). Using this procedure, all spectra were converted into unit vectors in *n*-dimensional space, and a pair of vectors was compared with a dot product calculation, which by definition includes the cosine of the angle between the two vectors, herein referred to as the cosine similarity score.

All MS data were also subjected to a dereplication workflow analysis against databases available in the GNPS platform (17 databases including NIH, MassBank spectral library, ReSpec spectral library among others) using the following parameters: score threshold 0.5, minimum intensity peak 50, parent mass tolerance 2.0 Da, ion tolerance 0.5 Da, minimum matched peaks 6 and filter standard deviation intensity of 2.0.

A molecular networking was created using the online workflow at GNPS. The data was filtered by removing all MS/MS peaks within ± 17 Da of the precursor m/z. MS/MS spectra were filtered by choosing only the top 6 peaks in the ± 50 Da window throughout the spectrum. The data was then clustered with MS-Cluster with a parent mass tolerance of 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da to create consensus spectra. Further, consensus spectra that contained less than 2 spectra were discarded. A network was then created where the edges were filtered to have a cosine score above 0.5 and more than 6 matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top-10 most similar nodes. The spectra in the network were then searched against the GNPS's spectral libraries. The spectra databases were filtered in the same manner as the input data. All matches kept between network spectra

and library spectra were required to have a score above 0.5 and at least 6 matched peaks.

Mass spectrometry data of all EtOAc bacterial extracts used in this study were added to the Public Massive datasets in GNPS (Massive ID MSV000078947).

4.5 NMR analysis

All spectra were recorded on a Bruker DMX 300 spectrometer [at 300 MHz (¹H) and 75 MHz (¹³C)] and 500 cryo-spectrometer [at 500 MHz (¹H) and 125 MHz (¹³C)] using adequate deuterated solvents. Chemical shift values were referenced to residual solvent signals for CDCl₃ (δ_H/δ_C, 7.21/77). HSQC, HMBC, COSY or TOCSY data were recorded using a Bruker DMX 500 cryo-spectrometer instrument. NMR data were processed using the MestReNova version 1.3 software.

4.6 HRMS measurements

HRMS measurements for exact mass determination were performed on a MICROMASS Zabspec TOF spectrometer for electrospray ionization at the CRMPO (Centre Régional de Mesures Physiques de l'Ouest), University of Rennes 1.

4.7 Characteristics of isolated compounds

Cyclo-(L-Leu, L-Pro) (**13**): white powder; *R*_f 0.38 in CH₂Cl₂/EtOAc/MeOH (15/15/3); t_R 18.15 min in H₂O/acetonitrile with 0.1% of HCOOH in the both solvents (cf. 4.4 in Experimental section); ¹H-NMR and ¹³C-NMR data (CDCl₃, 125 MHz) as described in the literature (Rui et al., 2013); HRESIMS *m/z* 233.126057 [M + Na]⁺ (calcd for C₁₁H₁₈N₂O₂Na, 233.124952).

6-methoxy 2-methyl-3-heptylprodiginin (**26**): pink powder; *R*_f 0.73 in CH₂Cl₂/EtOAc (95/5); t_R 41.72 min in H₂O/acetonitrile with 0.1% of HCOOH in the both solvents (cf. 4.4 in Experimental section); ¹H-NMR and ¹³C-NMR data (CDCl₃, 125 MHz) as described in the literature (Soliev, 2012) HRESIMS *m/z* 352.2384 [M + H]⁺ (calcd for C₂₂H₃₀N₃O, 352.23889).

4.8 Biological assays

Cytotoxic properties of extracts and pure compounds were determined with a standard tetrazolium assay (Millot et al., 2009). For the total EtOAc organic extracts of MOLA1416 and compound **26**, B16 cells were seeded at 15 000 cells/mL and HaCaT cells were seeded at 17 000 cells/mL in RPMI1640 medium with 5% fetal calf serum (FCS) at day 0 in well-plate. Cell lines were incubated at 37 °C in an atmosphere of 5% CO₂ during 24 h. After this time, pure compounds or crude extracts were added and after 48 h of incubation, cell growth and viability were measured at 540 nm, using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Each experiment was repeated at least three times.

Acknowledgments

We gratefully acknowledge the INSA Rennes for the ministerial PhD fellowship of Delphine Parrot. This work was partly supported by the EMR, a partnership between the UPMC, the Laboratoires Pierre Fabre and CNRS and by the project MALICA (10-INBS-02-01) of the Agence Nationale de la Recherche, France. We thank the Bio2Mar platform for access to its instrumentation and facilities. We also thank Aurelie Sauvager, Isabelle Rouaud (University of Rennes 1, PNSCM team) and Clémence Rohée, Cécile Vilette (Banyuls-sur-Mer) and Eric Hitti (University of Rennes 1) for their technical help. Dr. Nicolas Gouault (University of Rennes 1, PNSCM team) for the synthesis of the two generic compounds used for the mass parameters. Dr Sylvain Guyot, Hélène Sotin for P2M2 platform access. We thank the CRMPO center (Centre Rennais de Mesures Physiques de l'Ouest) for HRMS analysis and Dr Arnaud Bondon and Sandrine Pottier (PRISM-BioGenOuest, Plate-forme rennaise d'imagerie et spectroscopie multi-modales) for NMR material access.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.phytochem.2017.10.005>.

Uncited reference

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Appendix A. Supplementary data

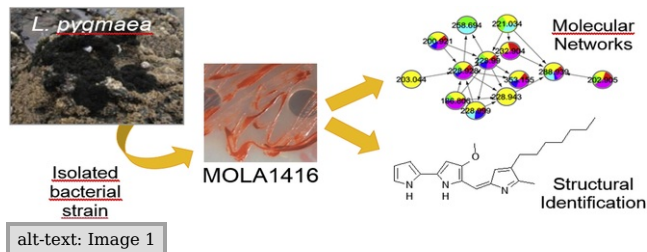
The following is the supplementary data related to this article:

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mmc1

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Graphical abstract



Highlights

- Chemical investigation of a Rhizobiales strain associated with *Lichina pygmaea*.
- Dereplication workflow based on MS/MS signatures and molecular networks were used.
- 25 putative known compounds were revealed.
- 2 metabolites were isolated and structurally identified.

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