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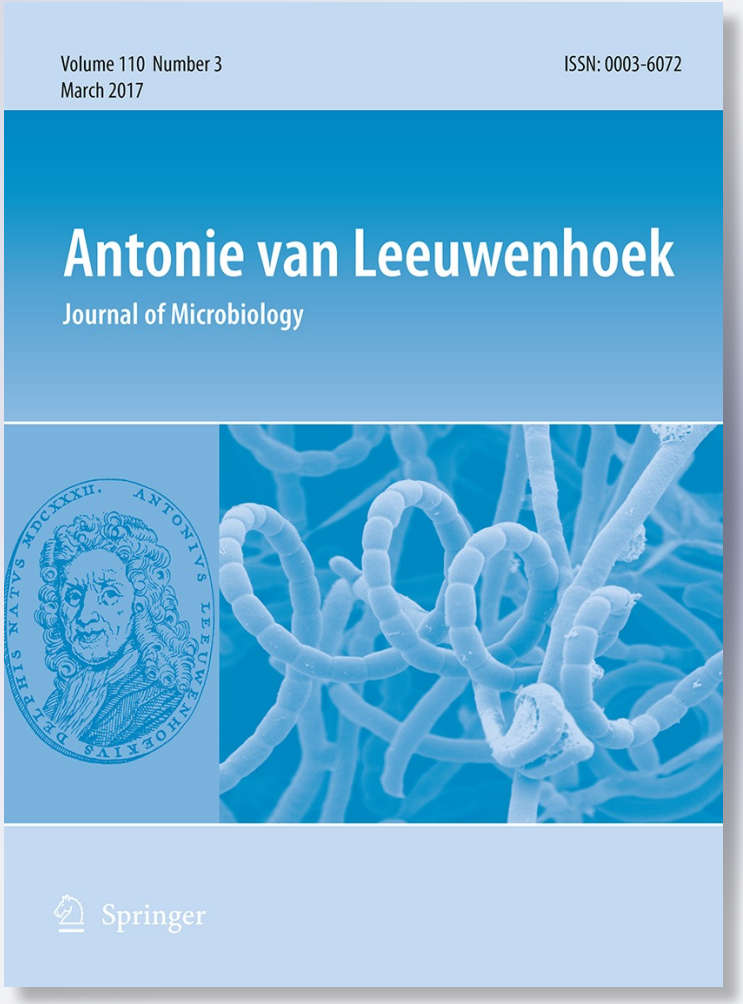
Antonie van Leeuwenhoek
Journal of Microbiology

ISSN 0003-6072
Volume 110
Number 3

Antonie van Leeuwenhoek (2017)
110:415-428
DOI 10.1007/s10482-016-0814-2



Springer

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ORIGINAL PAPER

Formation of indigoidine derived-pigments contributes to the adaptation of *Vogesella* sp. strain EB to cold aquatic iron-oxidizing environments

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Received: 16 June 2016 / Accepted: 26 November 2016 / Published online: 3 December 2016

Abstract We investigated previously under explored cold aquatic environments of Andean Patagonia, Argentina. Oily sheens similar to an oil spill are frequently observed at the surface of water in creeks and small ponds in these places. Chemical analysis of a water sample revealed the occurrence of high concentrations of iron and the presence of a free insoluble indigoidine-derived pigment. A blue pigment-producing bacterium (strain EB) was isolated from the water sample and identified as *Vogesella* sp. by molecular analysis. The isolate was able to produce indigoidine and another derived-pigment (here called cryoindigoidine) with strong antifreeze properties. The production of the pigments depended on the cell growth at cold temperatures (below 15 °C), as well as on the attachment of cells to solid surfaces, and iron limitation in the media. The pigments produced by strain EB showed an inhibitory effect on the growth of diverse microorganisms such as *Candida albicans, Escherichia coli* and *Staphylococcus aureus*. In addition, pigmented cells were more tolerant to freezing than non-pigmented cells, suggesting a role of cryoindigoidine/indigoidine as a cold-protectant molecule. The possible roles of the pigments in strain EB physiology and its interactions with the iron-rich environment from which the isolate was obtained are discussed. Results of this study suggested an active role of strain EB in the investigated iron-oxidizing ecosystem.

Keywords Aquatic environments · Cold temperatures · Cryoindigoidine · *Vogesella*

Electronic supplementary material The online version of this article (doi:10.1007/s10482-016-0814-2) contains supplementary material, which is available to authorized users.

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Introduction

Patagonia is an enormous region located in south Argentina, which contains a diversity of wild land terrestrial ecosystems consisting of extensive eastern semiarid areas bordering the Andes and cold subantartic zones at the south of the region. Natural forest ecosystems occur in Andean Patagonia in the West.

The western areas are covered by a dense Valdivian forest, with fertile valleys with humid-austral climate and extensive glaciers and peaks of the highlands. Winters are usually severe in this region. The high rainfall against the western Andes and the low surface temperatures give rise to cold and humid air masses, contributing to the ice-fields and glaciers. Cold waves can bring cold periods, and temperatures can fall to under −21 °C and remain below freezing for several days. In spite of these harsh and challenging conditions, the humid ecosystems of Andean Patagonia are expected to have a broad microbial biodiversity, which may be adapted to these conditions. However, they are considered as little-explored habitats and most of microbial activities in such environments remain to be investigated. Only few studies on some members of the microbial communities of humid ecosystems in Andean Patagonia have been previously reported (Castillo et al. 2007; Brandão et al. 2011; Tognetti et al. 2013).

Reddish-deposits, probably of iron hydroxides, can be frequently observed in superficial bodies of water in the region that surrounds the 42th Southern Parallel (El Bolsón and Lago Puelo Villages) and further south to Tierra del Fuego (Fig. S1). The abundance of bacteria able to metabolise iron leaching from the ground, which is rich in pyrite in that area, is probably responsible for these deposits. There are several bacteria that grow and multiply in water and oxidize dissolved iron into its insoluble ferric state (rust) and produced fluffy deposits that surround their cells, as part of their metabolism (Emerson and Revsbech 1994; Duckworth et al. 2009; Sawayama et al. 2011). An oily sheen on the water surface that reflects a rainbow of colours (principally blue) frequently occurs (Fig. S1). The oily sheen can look like an area where motor oil has been spilled. However, this sheen results from natural sources. The exact origin of this oily sheen is unknown although there are some presumptions about its cause. It is presumed that this substance contains iron and manganese inorganic compounds produced by 'iron bacteria' as by-products from their metabolic processes (http://des.nh.gov/organization/commissioner/pip/newsletters/sampler/documents/2015may.pdf). An alternative assumption considers an organic origin of the oily sheen on the water surface caused by the decomposition of aquatic insects and/or breakdown of dead iron bacteria or algal cells (http://pubs.usgs.gov/gip/microbes/index.html#anchor48513). To our knowledge, the exact composition and origin of the oil-like film on surface of quiet water observed in West Patagonia and similar ecosystems remains to be investigated in detail.

In order to contribute to knowledge of the biology and chemistry of aquatic environments of Andean Patagonia, we investigated the occurrence of insoluble organic compounds of bacterial origin, within the oily sheen observed in aquatic environments of the region.

Indigoidine is a member of the class of pyridones that is a dimeric blue pigment synthesised from L-glutamine by different bacteria, such as *Vogesella indigofera* (Kuhn et al. 1965; Grimes et al. 1997), *Dickeya dadantii* (Reverchon et al. 2002), *Phaeobacter* sp. (Cude et al. 2012), *Streptomyces lavendulae* (Takahashi et al. 2007), among others. Two redox states of indigoidine have been reported, an oxidized blue form known as indigoidine, and a reduced colourless form referred to as leucoindigoidine (Kuhn et al. 1965; Heumann et al. 1968). Indigoidine is possibly synthesized in the oxidized form and then reduced by an oxalocrotonate tautomerase enzyme encoded by *igiF*, although this has not been experimentally confirmed (Cude et al. 2012). Indigoidine production by bacteria may provide the microorganism with a competitive advantage in the environment. In this context, different physiological functions have been proposed for indigoidine, including as antioxidant molecules which help to protect cells against oxygen radicals; as possible intracellular signalling molecules which contribute to modulation of swimming motility, among other cellular processes (Cude et al. 2012); or as antimicrobial agents which suppress the colonisation of competing organisms in the environment (Reverchon et al. 2002, Cude et al. 2012).

In this study, we isolated and characterised a bacterial strain belonging to genus *Vogesella* genus, which was able to produce a novel indigoidine-derived blue pigment, which was induced by cold temperatures, cell attachment to solid supports, and iron limitation. On the basis of the results obtained, we performed a structure–function correlation of the blue pigment produced by strain EB, and integrated the studied trait with the nature of the environment from which the isolate was obtained.

Materials and methods

Sample and bacterial isolation

The strain *Vogesella* sp. EB used in this study was isolated from a superficial water sample containing an oily sheen at the surface. The sample was collected from a water pond in the region of the 42th Southern Parallel in Andean Patagonia, Argentina (41°57′53″S and 71°32′03″W). The temperature of the sample was 10 °C. The sample was taken with a sterile plastic collector and was transported on ice at all times. The water sample was chemically analysed in the Environmental Laboratory of Oil M&S SA (Cañadón Seco, Santa Cruz, Argentina). Nutrient Broth (NB) agar plates were inoculated with 100 µl of the water sample and 100 µl of a dilution of the sample (1/10, v/v) in sterile saline solution (0.8% NaCl). Agar plates were incubated for 5–7 days at 28 °C. NB agar was used for the purification of the growing strains. *Vogesella* sp. strain EB has been deposited in the Banco Nacional de Microorganismos (Argentina) (BNM543).

Media and culture conditions

For growth experiments, cells were cultivated aerobically at 10, 15, 20, or 28 °C in NB medium supplemented with 10 g glycerol l^{-1} on a rotary shaker. Solidified medium was obtained by adding 2% (w/v) of agar–agar.

For blue pigment production, *Vogesella* sp. strain EB was cultured at 10 °C in NB medium on a rotary shaker, or grown 28 °C by immobilisation with agar beads (Nigam et al. 1998) in NB medium, or was grown at the surface of cellulose nitrate filters (Sartorius, Göttingen, Germany) (Alvarez et al. 2004; Bequer Urbano et al. 2013) placed in liquid NB medium in a plate.

To study the relationship between pigment production and iron concentrations, cells were grown aerobically at 28 °C in NB medium supplemented with 10 g glycerol l^{-1} on a rotary shaker. After growth, cells were harvested, washed twice with sterile NaCl solution (0.85%, w/v) and resuspended at an OD_{600} of 1 in sterile NaCl. Serial dilutions aliquots of 10 µl were spotted onto NB agar plates supplemented with 10 g glycerol l^{-1} of, in the presence of different concentrations of iron (14 and 42 mg ammonium ferric citrate l^{-1}). For this test, we also used NB medium supplemented with glycerol (10 g l^{-1}) prepared with water from the sampling place (AB medium).

Genome sequencing, annotation and analyses

Sequencing was performed by the commercial services of INTA Castelar (Argentina) using 454 GS FLX Titanium technologies (Margulies et al. 2005) and Assembly Program Newbler v2.6. The genome of strain EB contains 3540,160 bp, with 3194 proteins and 64.4% G+C. The sequences were submitted to NCBI with RefSeq (NZ_LFDT00000000.1/NZ_LFDT01000021.1). For sequence analyses, we used the diverse databases: KEGG, UniProt, Pfam, BLAST.

Taxonomic characterization and phylogenetic analyses of the isolate

Sequences were screened against the NCBI database using the BLAST search program. For phylogenetic analyses, sequences were aligned using the T-Coffee method (Notredame et al. 2000) and processed by the Genedoc program. Evolutionary trees were inferred using maximum-likelihood (Felsenstein 1981), maximum-parsimony (Kluge and Farris 1969) and neighbour-joining (Saitou and Nei 1987) methods. The resultant tree topologies were evaluated by carrying out bootstrap analyses (Felsenstein 1985) based on 1000 resamplings, using the SEQBOOT, DNADIST and CONSENSE programs in the PHYLIP package (Felsenstein 1993).

Pigment extraction and chemical analyses

Cells were grown in NB supplemented with 1 g glycerol l^{-1} on a rotary shaker for 72 h at 10 °C. After growth, cells were harvested, washed two times with NaCl solution (0.85%, p/v), and once each with distilled water and chloroform, respectively. The pigment was extracted from cells by addition of dimethyl sulfoxide (DMSO) (1:1, p/v) in two sequential steps.

Visible spectral analyses of the extracted pigment in DMSO were recorded on a Thermo Scientific GENESYS 20 Visible Spectrophotometer (Takahashi et al. 2007, Yu et al. 2013).

To compare the Infrared spectra of the blue pigment and the oily sheen sample water, both samples were adjusted to a final mix of DMSO: water (1:1) and run in a solid state potassium bromide tablet in a frequency range between 200 and 6800 cm^{-1}. Measurements

were made in a Perkin Elmer IR spectrum and compared to its software database.

For HPLC analysis, the pigment dissolved in DMSO was filtered through a 0.22 μm Teflon/Prefilter and analysed on a Waters 1525 HPLC (C18, 5 μm, 4.6 × 250 mm Waters Symmetry 300 column), eluted with a linear gradient of 10–90% aqueous methanol over 25 min at a flow rate of 1 ml min^{-1} (Yu et al. 2013). The equipment had a diode-array detector Waters 2996 and refractive index detector (used the range 200–800 nm).

For IR analysis, IR spectra of the purified pigment in DMSO were measured on a Nicolet Magna 550 FT-IR spectrophotometer in solid state (KBr cell, 0.2 mm length), in a frequency range between 450 and 4000 cm^{-1}.

For the determination of the molecular mass by ESI (electrospray) mass spectrometry, the purified pigment was dissolved in DMSO: MeOH and analysed using a Bruker micrOTOF-Q II 10193 mass spectrometer in positive mode.

For nuclear magnetic resonance (NMR), the purified pigment was dissolved in DMSO. All 1D and 2D NMR spectra (HSQC and HMBC) were recorded on a Magnetic Resonance Nuclear Bruker Advance II 500 spectrometer (1H to 500.13 MHz; 13C to 125.77 MHz). The processing software was Topspin 2.1 (Bruker) in DMSO solution; δ in ppm rel. to Me_4Si as internal standard, J in Hz.

Iron oxidation test

For the iron oxidation test, four NB semisolid agar tubes were prepared, two of them containing 10 mM nitrate, another containing 2 mM $FeCl_2$ and the last one containing 10 mM nitrate and 2 mM $FeCl_2$. Cells of strain EB were grown on NB plates supplemented with 10 g glycerol l^{-1} for 3 days. The colonies were used to inoculate the iron oxidation test tubes, which were then covered with a Vaseline layer and closed with rubber caps and film to ensure anoxic conditions (Chan et al. 2011). After 4 weeks of incubation at 28 °C in the darkness, one tube containing nitrate was supplemented with 3 ml $FeCl_2$ (40 mM) and covered with Vaseline again. After incubation at 28 °C in the darkness, positive tubes for iron oxidation were identified by the presence of a brownish-green or brownish-red precipitate (Weber et al. 2006). Reduction of nitrate was analysed by the Griess Test (Sun et al. 2003), with aliquots taken from the middle of each test tube.

Antimicrobial susceptibility testing

Firstly, the turbidity of microbial suspensions (*Escherichia coli*, *Rhodococcus opacus*, *Staphylococcus aureus, Candida albicans* and *Rhodotorula* sp.) was adjusted to 0.5 McFarland Units. The bacterial suspensions were spread on agar plates of LB for *E. coli*, *S. aureus* and *R. opacus*, and NB with 1% (w/v) glucose for *C. albicans* and *Rhodotorula* sp. After that, 5 μl of a suspension of strain EB was inoculated in the centre of each plate, which were incubated at 25 °C for 2 days, as reported previously by Cude et al. (2012).

Survival at freezing temperatures

Cells were grown aerobically at 28 °C overnight in 10 ml NB medium on a rotary shaker. After growth, cells were harvested, washed once with sterile NaCl solution (0.85%, w/v) and resuspended in sterile NaCl until a dilution 1:9 gave an OD_{600nm} of 0.2. This cell suspension was used to inoculate NB agar plates containing 10 g glycerol l^{-1} to produce blue cells and liquid NB medium with 10 g glycerol l^{-1} to produce non-pigmented cells. NB agar plates were incubated for 72 h at 28 °C, whereas liquid NB medium was incubated for 72 h at 28 °C on a rotary shaker. After growth, both sets of cells were harvested, washed twice with sterile saline solution (0.8% NaCl) and resuspended in Mineral Salts Medium (MSM) containing 0.1 g ammonium chloride l^{-1} and 1% (w/v) of glucose at an OD_{600nm} of 0.9 of a 1:9 dilution. Aliquots from this cell suspension (1 ml each) were incubated at −15 °C for 24 and 48 h. The survival rate was calculated as $CFU_{after\ treatment}/CFU_{before\ treatment} \times 100$. Here, we related the term "survival rate" with the ability of cells to resume growth after plating, and not to differentiate between viable and dead cells. All CFU determinations were done in triplicate experiments. The data were recorded as means and standard deviations.

Results

Characterisation of the ecosystem associated with the occurrence of the oily sheen

An oily sheen at the surface of quiet water has often been observed in aquatic habitats of Andean Patagonia

(Fig. S1). This blue iridescent insoluble material was frequently associated with the occurrence of a reddish-brown fluffy material on the rocks and reeds in the bottom of creeks or ponds (Fig. S1). The appearance of these ecosystems is indicative of the iron bacteria activity, which produces insoluble ferric compounds by oxidation of dissolved iron in water. In order to characterise the iron-oxidizing ecosystem, water samples were collected for chemical and microscopic analyses. Chemical analyses of samples revealed high concentrations of iron (3.8 mg l^{-1}) and neutral pH in the surface water. Microscopic analyses of bacteria collected from the reddish-slimy material and surface water revealed the occurrence of flocs formed by the association of bacterial and algal cells, and a fluffy material (Fig. 1a). Among the bacterial cells observed, some cells with a long filamentous morphology compatible with iron-oxidizing bacteria of the group *Leptothrix* were detected. Figure 1b, c shows filamentous cells with inorganic precipitates attached to the sheath. The products of the oxidative reactions, probably ferric hydroxide or ferric oxide, are usually chelated to organic substances, and sometimes coat the sheaths of the bacteria. Taken together, the occurrence of reddish-brown slimy deposits in aquifers, the high concentration of iron in the water, and the occurrence of long filamentous cells with inorganic precipitates attached to the sheath are consistent with the designation of these aquifers as iron-oxidizing ecosystems.

Characterisation of strain EB isolated from an aquifer

A bacterial strain, designated EB, able to produce a blue pigment was isolated from a water sample collected in an aquifer (Fig. 1d). Old colonies of strain EB became iridescent blue (Fig. 1e). After addition of water to the surface of the nutrient agar, an insoluble blue sheen was released from the colonies (Fig. 1f). Cells of strain EB were found to be Gram-negative rods, oxidase positive and to produce polyhydroxybutyrate granules. A phylogenetic analysis based on the sequence of the 16S rRNA gene was performed to identify the isolate. This revealed that the strain was taxonomically close to *V. indigofera* (97% identity). Comparative analysis of the complete 16S rRNA gene sequence (Genbank Accession Number KJ792811) placed strain EB in the beta subclass of the Proteobacteria and in a close relationship with

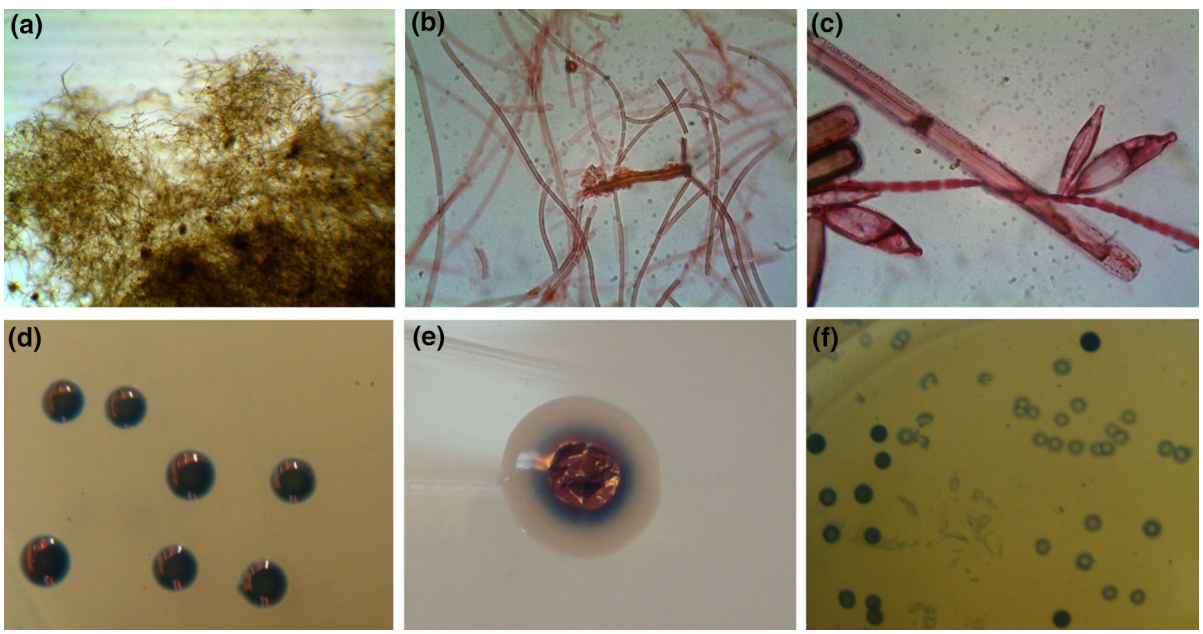


Fig. 1 **a–c** Optical microscopy of the reddish-slimy material and surface water from Andean Patagonia samples. Young **d** and old **e** colonies of strain EB grown on NB agar plates added with glycerol 1% (v/v), after 24 h and 7 days of incubation at 28 °C, respectively. **f** Release of the insoluble pigment from the colonies after adding water at the surface of the solid medium

Vogesella spp., *Chromobacterium violaceum*, *Lutiella nitroferrum* and *Laribacter hongkongensis* (Felsenstein 1981) (Fig. S2). *C. violaceum* and *L. nitroferrum* have been previously recognised for their ability to oxidize Fe(II) in a nitrate-dependent manner (Weber et al. 2006). For this reason and because strain EB was isolated from an iron rich environment, we compared the putative genes for nitrate reduction of EB with those of the nitrate-dependent iron-oxidizing *C. violaceum* and *L. nitroferrum* and investigated its ability to oxidize Fe(II) (Fig. S2a). Our sequence analysis indicated that the gene content and organization of the nitrate reduction gene clusters in strain EB are similar to those of *C. violaceum* and *L. nitroferrum*. The respiratory nitrate reductase cluster is similar between the three microorganisms, containing alpha, beta, gamma and delta chains and usually preceded by a nitrate/nitrite transporter. *L. nitroferrum* has a duplicated set of these genes located in a distinct place in its genome (Fig. S3a). The analysis of the *Vogesella* sp. EB protein sequences using the BLAST algorithm revealed a high identity (*I*, between 46 and 93%) with the nitrate reduction genes of *C. violaceum* and *L. nitroferrum* (Fig. S3a). The occurrence of a similar gene cluster for nitrate reduction in strain EB in comparison with those of the related nitrate-dependent iron oxidizing *C. violaceum* and *L. nitroferrum* may suggest a similar ability of strain EB to oxidize Fe(II) coupled to the reduction of nitrate to nitrite. For this reason, we analysed the capability of *Vogesella* sp. EB for growth in anoxic NB semisolid tubes and to oxidize Fe(II) in the presence of nitrate (Fig. S3b). Fe precipitate and nitrate reduction occurred only in the culture tube containing Fe(II) and nitrate (Fig. S3b). No nitrate reduction and Fe precipitation occurred when strain EB was cultivated in NB with nitrate or Fe(II) in separate tubes (Fig. S3b). These results suggested an active ecological role of bacteria such as *Vogesella* sp. in the iron-oxidizing aquatic ecosystems of Andean Patagonia.

Grimes et al. (1997) reported the isolation of bacterial strains belonging to the genus *Vogesella* from freshwater samples, with the ability to produce blue-pigmented colonies that had a metallic copper-coloured sheen. The blue pigment produced by those microorganisms has been identified as indigoidine (5, 5′-diamino-4, 4′dihydroxy-3, 3′-diazadiphenoquinone-[2, 2′]) (Fig. 2) (Weber et al. 2006). The blue pigment produced by strain EB was preliminary identified by scanning the visible spectrum absorbance and IR spectrometry. The absorption maximum of the blue pigment extracted with DMSO was 612 nm. In addition, the IR spectrum showed maximum frequencies associated with several groups: aromatic, amine and probable ketone groups (Fig. 3). These main groups are usually present in the indigoidine molecule (Grimes et al. 1997; Fig. 2).

Chemical analysis of the oily sheen collected from Andean aquifers

Considering that blue pigment-producing microorganisms seem to be members of the microbial communities of Andean aquifers, we investigated the occurrence of free indigoidine in the oily sheen collected at the surface of quiet water in the Andean Patagonia. The purified blue-pigment produced by strain EB was analysed in parallel with the environmental water sample. TLC analysis of the environmental sample revealed the occurrence of a spot with the same R_f value (0.52) as the blue-pigment produced by strain EB (data not shown). When comparing the IR analyses of the oily sheen sample and the extracted pigment, both of them exhibited identical spectra as is shown in Fig. 3. These results demonstrated the occurrence of free indigoidine in the oily sheen collected at the surface of aquifers in the Andean Patagonia.

Key genes for indigoidine metabolism

Sequencing the draft genome of strain EB (RefSeq NZ_LFDT00000000.1/NZ_LFDT01000021.1) provided the opportunity to perform a genome-wide examination of

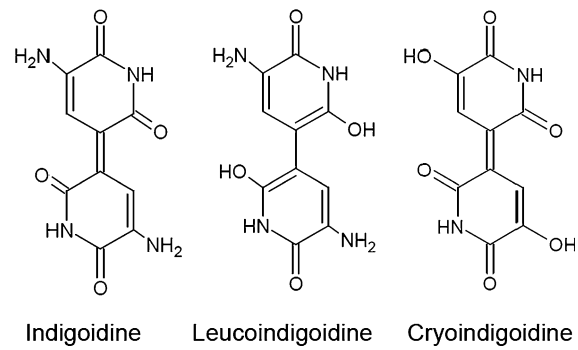


Fig. 2 Chemical structures of indigoidine, leucoindigoidine and cryoindigoidine [(3*E*)-5-hydroxy-3-(5-hydroxy-2,6-dioxo-1,6-dihydropyridin-3(2*H*)-ylidene)pyridine-2,6(1*H*,3*H*)-dione ($C_{10}N_2O_6H_6$)]

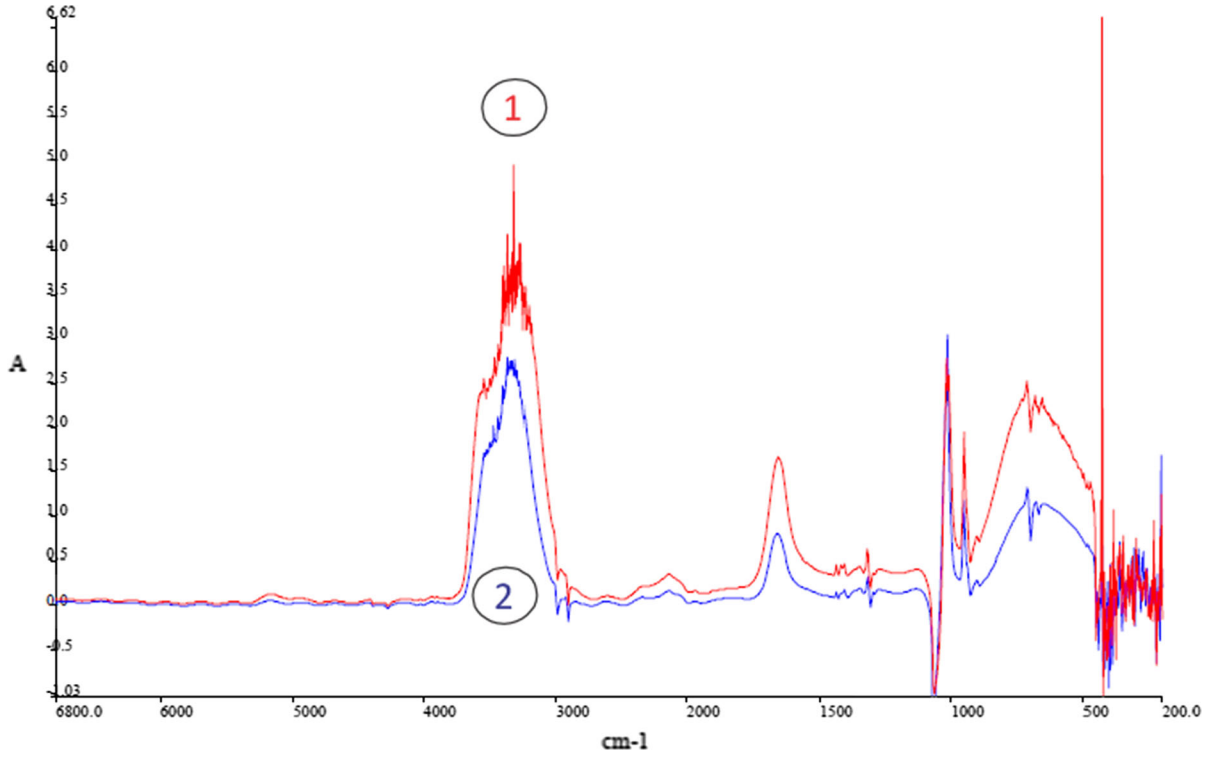


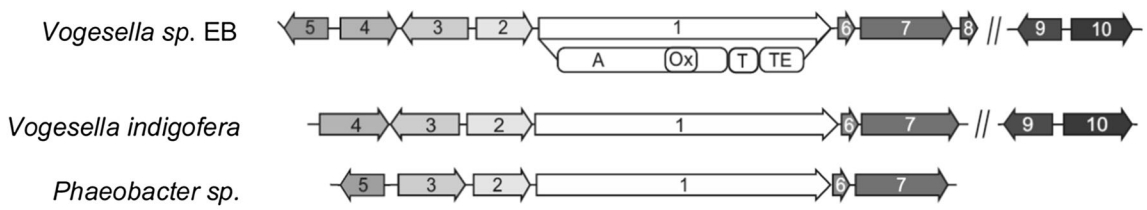
Fig. 3 IR-spectrum suggesting the presence of cyclical ketone; amine, hydroxyl group and amide substituent. *1* Water, *2* EB Pigment. *A* Absorbance

key genes involved in indigoidine metabolism. This pigment is synthesised by condensation of two units of L-glutamine by a 4′-phosphopantetheinyl-transferase-activated NRPS (Reverchon et al. 2002; Takahashi et al. 2007). The degree of identity of full protein sequences of different indigoidine-synthesising bacteria is shown in Fig. 4b. *Vogesella* sp. EB exhibits similar gene organisation when compared with *V. indigofera* and *Phaeobacter* sp. Y4I (Takahashi et al. 2007; Cude et al. 2012), as is shown in Fig. 4a. The major cluster includes a TetR transcriptional regulator, a phosphopantetheinyl transferase (IgiA), a hydroxyisobutyrate dehydrogenase (IgiB), an arylmalonate decarboxylase (IgiC), an indigoidine synthase (IgiD), a tautomerase (IgiF), a MFS transporter (IgiE), and a hypothetical protein (IgiG). In a separate locus, we found the genes encoding the permease PecM and the transcriptional regulator PecS, which are both related to indigoidine production, as has been reported for *Dickeya dadantii* (Reverchon et al. 2002; Rouanet and Nasser 2001; Chu et al. 2010) (Fig. 4b).

The main enzyme involved in the pigment biosynthesis is a NRPS called indigoidine synthase, which includes four domains: (1) an adenylation domain (A, 39–486 and 887–927 aa), (2) an oxidation domain (Ox, 590–701 aa), (3) a thiolation domain, which attachs 4′-phosphopantetheine prosthetic group (T, 938–1017 aa), and (4) a thioesterase domain (TE, 1039–1230 aa) (Finking and Marahiel 2004). Interestingly, we found in the strain EB NRPS a conserved domain similar to an enterobactin synthase (EntF) (21–455 and 888–1134 aa), which is a siderophore occurring in alphaproteobacteria (Reichert et al. 1992). The relationship of this domain to iron metabolism needs to be investigated in the future.

Conditions that promote pigment production by strain EB

We investigated the conditions that promote pigment production by strain EB. Cells were grown in liquid NB containing 10 g glycerol l^{-1} at 28, 20, 15 and 10 °C for 5 days (Fig. 5a). The blue pigment was produced only during cultivation of cells at 15 and 10 °C, but not at 28 °C and in reduced amounts at 20 °C (Fig. 5b). Pigment production seemed to be a



N°	*Vogesella sp.* EB	Enzyme	Abbreviation	*Vogesella indigofera*	*Rhodobacterales bacterium* Y4I	*Photorhabdus luminescens*	*Dickeya dadantii* 3937	*Streptomyces aureofaciens* CCM3239
10	ACG97_RS12145	Transcriptional regulator, MarR	PecS	Vind_07 97%		plu1277 28%	Dda3937_00976 39%	
9	ACG97_RS12150	Permease	PecM	Vind_06 98%		plu4591 29%	Dda3937_00975 41%	Sa3 50%
8	ACG97_RS12090	Hypothethycal protein	IgiG	98%	N/D	N/D	N/D	
7	ACG97_RS12085	Indigoidine transporter	IgiE	Vind_05 88%	RBY4I_RS01655 58%	plu1003 21%	Dda3937_02457 29%	
6	ACG97_RS12080	Tautomerase	IgiF	99%	RBY4I_RS01660 37%	plu2181 30%		
1	ACG97_RS12075	Indigoidine synthetase	IgiD/IndC	Vind_04 98%	RBY4I_RS01665 54%	plu2186 44%	Dda3937_00972 45%	sa8 47%
2	ACG97_RS12070	arylmalonate decarboxylase	IgiC	Vind_03 98%	RBY4I_RS01670 59%			
3	ACG97_RS12065	Hydroxyisobutyrate dehydrogenase	IgiB	Vind_02 99%	RBY4I_RS01675 52%			
4	ACG97_RS16980	Phosphopantetheinyl transferase	IgiA	Vind_01 97%	RBY4I_RS02855 39%	plu0992 32%	Dda3937_02667 33%	
5	ACG97_RS12055	Transcriptional regulator	TetR	Vind_00 97%	RBY4I_RS01680 39%			

Fig. 4 **a** Organization and **b** percentages of identity of genes putatively involved in indigoidine biosynthesis from strain EB and diverse pigment-producing bacteria. *A* adenylation domain, *Ox* oxidation domain, *T*, thiolation domain, *TE* thioesterase domain

temperature-dependent process during cultivation of cells in liquid media, since inducing osmotic stress and oxidative stress (by the addition of pro-oxidants as H_2O_2 and methyl viologen to the culture media) did not promote pigment production by strain EB during cultivation at 28 °C. However, cells of strain EB were able to produce indigoidine in liquid cultures at 28 °C, only when cells were attached to an inert support, such as cellulose nitrate filters or agar beads, or when they were grown as colonies on agar surfaces (Fig. 5c).

Since strain EB was isolated from an iron-rich aquifer, we analysed the possible influence of iron on pigment production. For this, cells were cultivated in NB broth with glycerol, in the absence or presence of different iron concentrations. In addition, cells were grown in the same medium prepared with aquifer water, from where the strain was isolated (Fig. 5d). The results suggested that the pigment production by strain EB was stimulated by the absence or low concentrations of iron in the media, as is shown in Fig. 5d. A high concentration of iron seemed to inhibit pigment production by cells (Fig. 5d).

Antifreeze properties of pigments produced by strain EB and chemical characterisation of pigments

During the sequential extraction and storage of the blue pigment produced by strain EB, we observed that the first DMSO extract (green–blue) did not freeze at −20 °C, whereas the second one (blue) did (Fig. 6a). Interestingly, the first DMSO extract did not freeze even after storage at −70 °C. To explain the differences between the DMSO extracts, we performed

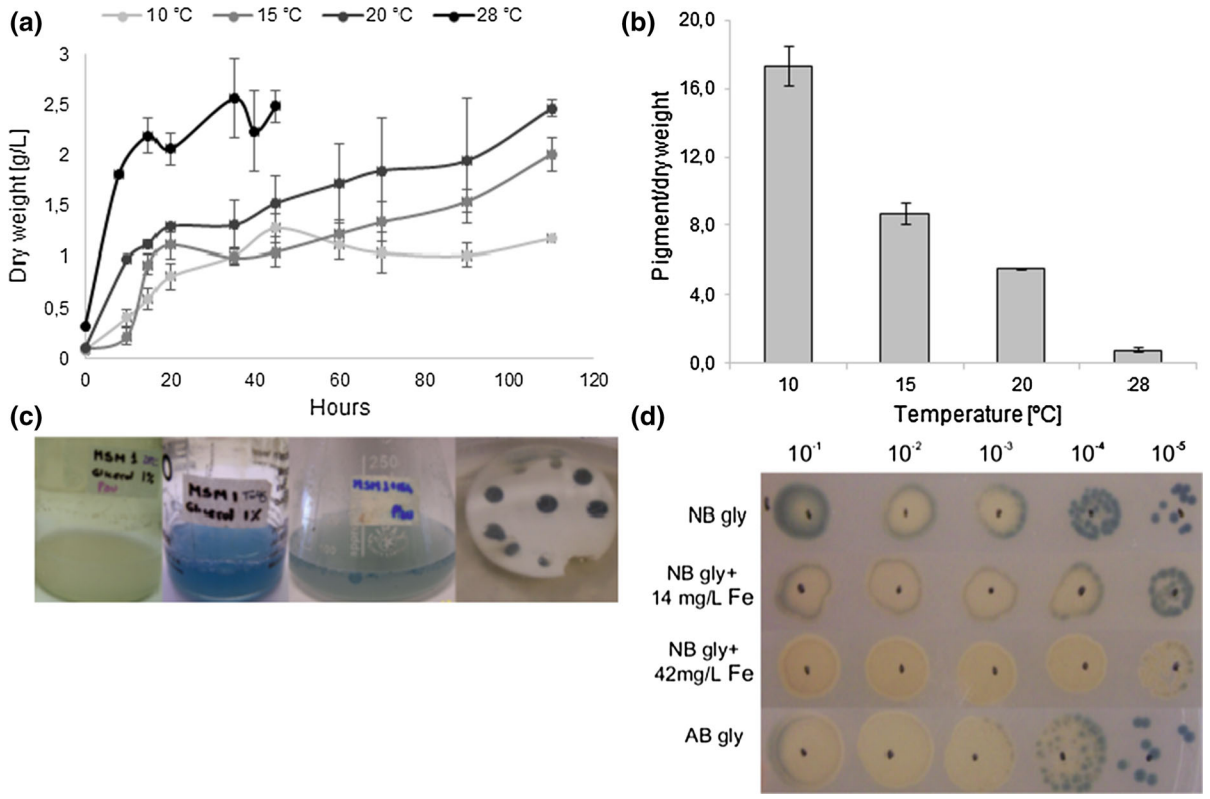


Fig. 5 **a** Growth kinetics of *Vogesella* sp. EB cultivated in NB medium with 10 g glycerol per l at the different temperatures. **b** Maximum production of pigment at different temperature of cultivation. **c** Pigment production by strain EB at 28 and 10 °C on liquid media; pigmented cells attached on agar beads at 28 °C, and colonies of strain EB attached at the surface of cellulose nitrate filters incubated at 28 °C (from *left* to *right*). **d** Growth and pigment production by EB cells in the presence of different concentrations of iron in the media. *NB* nutrient broth medium, *AB gly* medium prepared with aquifer water with 1 g glycerol l^{-1}

diverse chemical analyses to detect the occurrence of different pigment variants in the cells.

Crystallisation methods demonstrated that samples contained a mixture of two indigoidine-derived pigments that could not be separated (data not shown). Pigments from both samples were analysed by ESI–MS. A small peak at m/z 248.0 was obtained in the samples, which was consistent with the molecular weight of indigoidine ($C_{10}H_8N_4O_4$). Interestingly, the mass spectra (in both cases) showed a major peak m/z at 257.042 which was not compatible with those of indigoidine (248.0–249.02 m/z) (Fig. 6c) (Cude et al. 2012, Takahashi et al. 2007) or leucoindigoidine (251.0 m/z, Cude et al. 2012). These results suggested that the major pigment produced by strain EB possesses a structure derived from indigoidine, but with some modifications. IR spectra of pigments from both DMSO extracts showed maximum frequencies associated with several groups: hydroxyl group (3200–3400 cm^{-1}), secondary amide (3440–3300 cm^{-1}), cyclical ketone (1715 cm^{-1}), keto group (1680–1655 cm^{-1}), amine group (1530–1550 cm^{-1}), and C–N binding (1260–1300 cm^{-1}) and a medium-strong carbonyl absorption at 1730 cm^{-1} (Fig. 6b). These main groups are usually present in the indigoidine molecule (Grimes et al. 1997), with the exception of the hydroxyl (OH) group (Fig. 2). In addition, pigments from both samples were analysed by ^{1}H-NMR. The analysis showed the occurrence of peaks at 8.15 (NH), 5.3 (NH_2), 4.6 (OH), which are consistent with the reported spectrum of indigoidine (Cude et al. 2012, Takahashi et al. 2007), except for the OH groups. Finally, in the HSQC spectrum, signals at d(C) 136 and 130 indicated the presence of two C=C bonds, while the signal at d(C) 160 was assigned to a ketone group. All these results indicated that samples contained

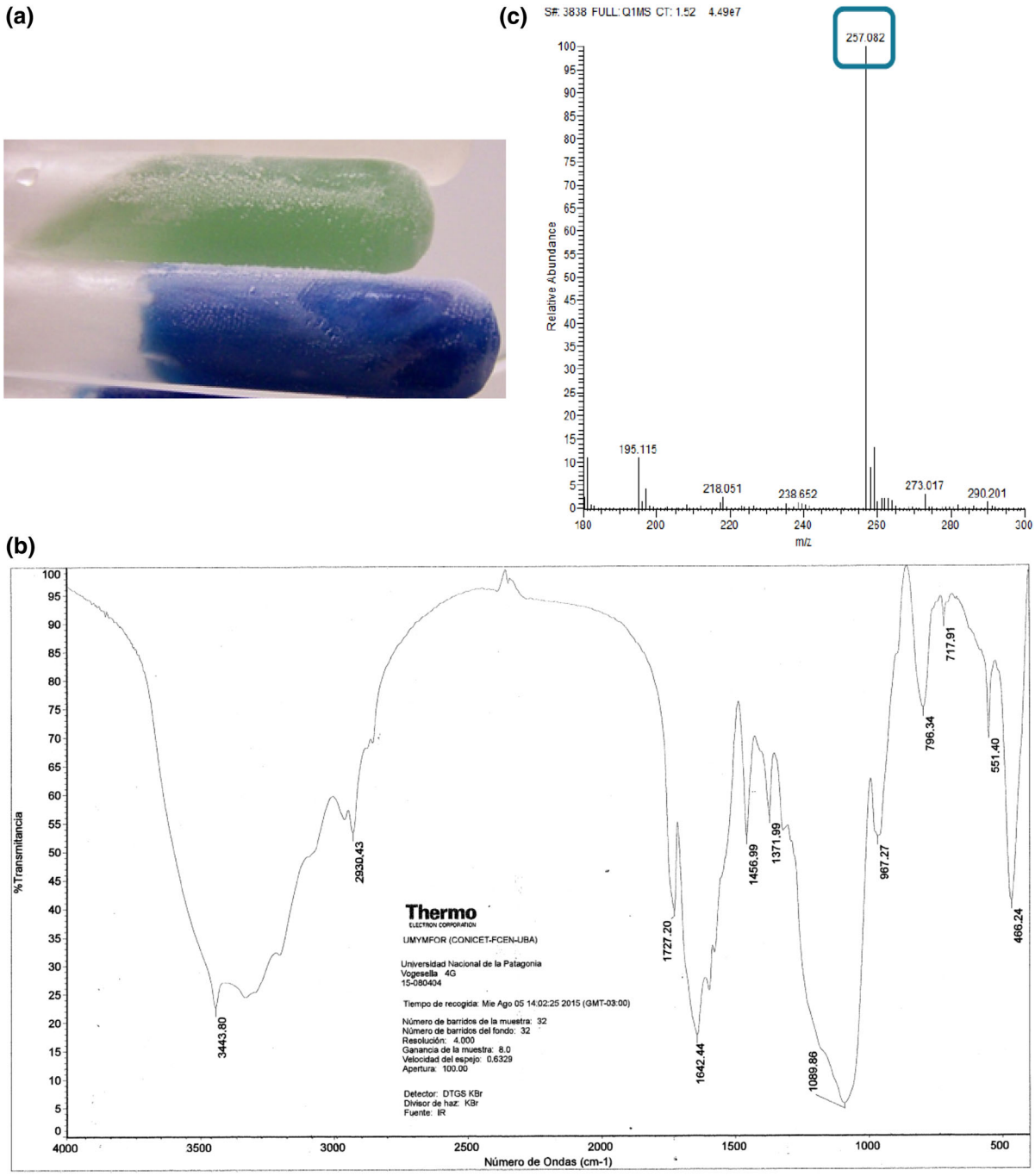


Fig. 6 **a** Image of the first and second DMSO extracts of the pigment produced by strain EB. **b** IR-spectra obtained after analyses of the pigment produced by EB cells. **c** Mass spectrum analysis by ESI–MS. *Note* that the m/z peak at 257.042 is not consistent with protonated indigoidine (248 m/z, Takahashi et al. 2007; 249.02 m/z, Cude et al. 2012) or leucoindigoidine (251 m/z, Cude et al. 2012)

indigoidine as a minor component and, in addition, another pigment variant different to indigoidine or leucoindigoidine. Based on the obtained results, we suggest that the novel blue pigment, which was present in both DMSO extracts, is (3*E*)-5-hydroxy-3-(5-hydroxy-2,6-dioxo-1,6-dihydropyridin-3(2*H*)-ylidene)

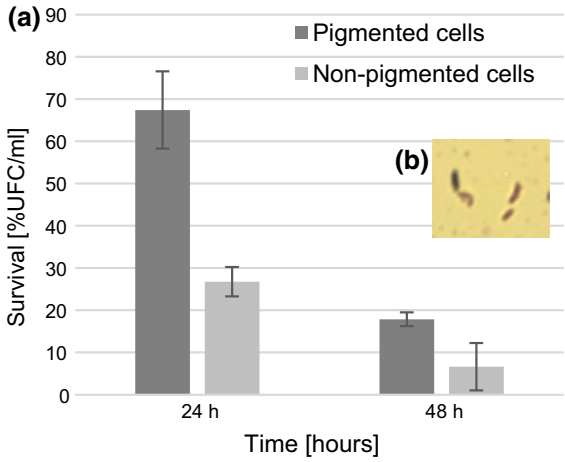


Fig. 7 **a** Tolerance of pigmented and non-pigmented cells of strain EB to freezing temperatures (−15 °C) after incubation during 24 and 48 h. **b** Optical microscopy of pigmented cells (×100)

pyridine-2,6(1*H*,3*H*)-dione ($C_{10}N_2O_6H_6$, 250 g mol^{-1}) (Fig. 2). We propose the term of "cryoindigoidine" for this new pigment due to its contribution to the antifreeze property and because its production by strain EB is stimulated by low temperatures.

Possible physiological functions of pigments produced by strain EB

Considering the average temperature of the sampling area and the antifreeze property of cryoindigoidine/indigoidine (at least under the tested conditions), we analysed a possible role of pigments in the tolerance of cells to low temperatures. The area where the water sample was collected usually undergoes freezing temperatures during the winter season. For this reason, we analysed the survival of pigmented and non-pigmented cells of strain EB after incubation at −15 °C. Blue pigmented cells were more resistant to freezing than non-pigmented cells as is shown in Fig. 7a. Interestingly, microscopic observations of pigmented cells showed a uniform distribution of blue pigments along the cell rather than concentrated in specific places (Fig. 7b). Based on this observation and as cryoindigoidine/indigoidine are water insoluble, we presume that the pigment is localised at the cell membrane/cell envelope level in strain EB.

The release of free pigments to the environment might have an ecological importance for pigment-producing microorganisms. Cude et al. (2012) suggested that the production of indigoidine may provide an ecological advantage for *Phaeobacter* sp. strain Y4I due to the ability of the pigment to suppress the growth of diverse microorganisms, such as *Vibrio anguillarum*, *Ruegeria lacuscaerulensis* and *C. albicans*. For this reason, we investigated the ability of indigoidine-producing *Vogesella* sp. strain EB to inhibit the growth of different microorganisms (Fig. S4). Indigoidine-producing cells of strain EB were able to inhibit the growth of *E. coli, C. albicans* and *S. aureus.* In contrast, pigmented cells were unable to inhibit growth of *R. opacus* and *Rhodotorula* sp. (data not shown).

Discussion

Oily-looking films are usually observed at the surface of pristine aquifers associated with iron-oxidizing ecosystems in the Andean Patagonia (Argentina). Due to our interest in the ecology of the Patagonian region and the biotechnological potential of the microbial biodiversity of this area, we investigated the occurrence of microbial insoluble organic compounds in the oily sheens observed at the surface of water in creeks and small ponds. We isolated from water samples of an aquifer a bacterial strain able to produce a blue-pigment, which was identified as *Vogesella* sp. strain EB. Results of this study suggested that strain EB might be included among the iron-oxidizing bacteria occurring in iron-oxidizing aquatic ecosystems of Andean Patagonia. However, the role of *Vogesella* sp. as iron-oxidizing bacteria in their natural environment remains to be investigated in the future.

Strain EB was able to produce and excrete to the extracellular medium blue pigments related to indigoidine. The ability to produce indigoidine was previously reported for *V. indigofera*, a closely related bacterium (Grimes et al. 1997), and for other bacteria belonging to different taxonomic groups, including *Dickeya dadantti*, *S. lavendulae*, *Streptomyces aureofaciens*, *Photorhabdus luminescens* and *Phaeobacter* sp, among others (Grimes et al. 1997; Reverchon et al. 2002; Cude et al. 2012; Takahashi et al. 2007; Finking and Marahiel 2004; Starr et al. 1966; Müller et al. 2012; Novakova et al. 2010; Brachmann et al. 2012). We found that *Vogesella* sp. strain EB is able to synthesise indigoidine as minor compound and a modified blue form of indigoidine (here named as

cryoindigoidine) under the conditions used in this study (Fig. 2). Cryoindigoidine seemed to inhibit freezing of DMSO extracts at −20 and −70 °C, probably by a strong decrease of the freezing point of the solvent. Freezing point is a colligative property of a solution, which depends on the concentration of the dissolved substance. This may be the reason why the pigment inhibits freezing in the first DMSO extract, but not in the second extraction. Its chemical structure possesses polar groups (NH, OH), which may promote a strong interaction with H_2O molecules in multiple combinations, which may produce inhibition of subsequent crystal layer growth of ice.

Grimes et al. (1997) reported that the blue pigment indigoidine was localised within the cytoplasmic membrane in *V. indigofera*. The uniform distribution of the pigment along the cell in *Vogesella* sp. strain EB supported this assumption. The versatile structure of cryoindigoidine may allow a good arrangement of the pigment in the membrane architecture, probably through the interaction of its polar groups with charged residues of the membrane components. According to our results, we could speculate that pigments protect somehow the membrane integrity against low or freezing temperatures from the environment. The excretion and accumulation of pigments in the extracellular milieu could have the same protective function for the microbial community to extreme low temperatures in the environment. The relationship between pigments and the low temperatures is also supported by two additional results in this study. In this context, the production of blue pigments by *Vogesella* sp. strain EB required growth at temperatures below 15 °C. The Patagonian Andes aquifers usually reach low temperatures, so strain EB may be well adapted to produce pigments under these conditions. Moreover, results of this study suggested that the blue pigments enhance the survival of strain EB cells under low and freezing temperatures. Thus, the biosynthesis of cryoindigoidine/indigoidine may be part of complex strategic survival mechanisms in strain EB which allow it to colonise and thrive in extreme cold environments, as are found in the sub-polar region of west Patagonia. In this context, Schloss et al. (2010) reported the production of a red pigment during growth of a cold Alaskan soil isolated strain of *Janthinobacterium lividum* at cool temperatures. These microorganisms may serve as good models to investigate mechanisms of cold-specific gene regulation.

The production of indigoidine by bacteria seems to be modulated in bacteria by multiple regulatory events, including cellular systems mediated by quorum-sensing molecules and regulatory proteins (PecS-PecM transcriptional regulatory system); and by environmental factors such as temperature, pH, cell attachment or nutritional requirements (Reverchon et al. 2002; Rouanet and Nasser 2001; Starr et al. 1966; Fujikawa and Akimoto 2011). In addition to cold temperatures, the synthesis of blue pigments by *Vogesella* sp. strain EB was also stimulated by the attachment of cells to surfaces, such as inert filters or agar surfaces, and by the limitation of iron in the growth media. In this context, Cude et al. (2012) demonstrated that the gene responsible for indigoidine production (a NRPS encoded by *igiD*) in *Phaeobacter* sp. strain Y4I was up-regulated when cells were grown as colonies on agar surfaces and in biofilms compared to growth in broth cultures. In contrast, the induction of indigoidine production in bacteria by the absence or low concentration of iron in the surrounding environment has not been reported yet. According to these results, the pigments produced by strain EB may also have a functional relationship with the adaptation of cells to iron-rich environments. The bioavailability of iron in many environments is limited by the very low solubility of Fe^{+3}, which is the predominant state of iron in aqueous, non-acidic, oxygenated environments, such as those found in Andean Patagonia. Bacteria release siderophores to scavenge iron from the environment and form soluble Fe^{+3} complexes that can be taken up by active transport mechanisms. We observed several similarities between siderophores and cryoindigoidine/indigoidine, including: (1) they are synthesised and secreted by bacteria in response to iron restriction; (2) they are associated to the cell envelope and secreted through the plasma membrane, requiring specific transport proteins; (3) they are usually synthesised from simple precursors, such as glutamine and other amino acids; (4) they usually possess cyclic structures and highly polar residues; and (5) they are usually synthesized via NRPS's (Andrews et al. 2003). Moreover, we found in the strain EB NRPS the occurrence of a conserved domain similar to the module EntF in enterobactin synthase, involved in the synthesis of a siderophore widespread in alphaproteobacteria (Reichert et al. 1992). Altogether, these results suggested that cryoindigoidine/indigoidine could be involved somehow

in the iron cycling in *Vogesella* sp. EB. Whether cryoindigoidine/indigoidine can act as a siderophore-like compound or play a role in iron metabolism must be investigated in the future.

Taken together, the production of cryoindigoidine/indigoidine by strain EB seemed to be dependent on diverse factors, such as cold temperatures, iron restriction, cell attachment, among other factors. These factors may frequently occur in natural aquatic environments of Andean Patagonia, from which this strain was isolated, suggesting that blue pigment microbial production may be naturally stimulated. The ability of strain EB to release cryoindigoidine/indigoidine to the extracellular medium and, more interestingly, the occurrence of free blue pigments in the oily sheens observed at the surface of water in creeks and small ponds in Andean Patagonia, raises interesting questions about the physiological and ecological role of the pigments in this microorganism. The *de novo* biosynthesis of the pigments is probably an energetically expensive process, which competes for metabolic intermediates of the primary metabolism, as glutamine serves as precursor for the production of indigoidine pigments (Reverchon et al. 2002). Thus, the secretion of cryoindigoidine/indigoidine should represent an ecological advantage for the producing microorganism. In this study, we observed that the pigment-producing strain EB was able to inhibit the growth of diverse microorganism, such as *C. albicans*, *E. coli* and *S. aureus*. Thus, the results of this study suggest that *Vogesella* sp. strain EB may be an active component of iron-oxidizing communities in aquifers of Andean Patagonia, with the ability to synthesise and release cryoindigoidine/indigoidine into the environment under the prevailing conditions, which may contribute to the reduction of colonisation by other organisms. The occurrence of free indigoidine in the oily sheens observed at the surface of aquifers may control the diversity and composition of microbial communities in these iron-oxidizing environments. In addition, the accumulation of pigments in cellular membranes, as well as in the extracellular milieu, may protect cells against extreme cold temperatures that usually occur in water aquifers of Andean Patagonia. The protection mechanism of these pigments against extreme cold remains to be investigated.

This study contributes to a better understanding of the biology and chemistry in aquatic environments of Andean Patagonia, and of the identity and functions of microorganisms collected from these environments. In addition, further genome analysis of *Vogesella* sp. strain EB may provide in the future relevant information on the ecological and biotechnological properties of this microorganism.

Acknowledgements The authors thank the technical assistance of Dr. Gerardo Burton (University of Buenos Aires, Argentina) for the chemical analyses of the pigments and their interpretations. We also acknowledge Dr. Miguel Harvey (University of Patagonia SJB, Argentina) for crystallization analyses and discussions, and to Dr. Jorge Jaramillo Cisterna (University Austral of Chile) for fruitful discussions about results of chemical analyses. This study was financially supported by the SCyT of the University of Patagonia San Juan Bosco, and Project PICT2012 Nro. 2031 (ANPCyT), Argentina. Arrúa Day P. was recipient of a doctoral scholarship from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. Alvarez H.M is a career investigator of CONICET.

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