FEMS Microbiology Ecology 50 (2004) 163–173

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Bacterial diversity in Adélie penguin, *Pygoscelis adeliae*, guano: molecular and morpho-physiological approaches

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Received 4 March 2004; received in revised form 16 June 2004; accepted 21 June 2004

First published online 23 July 2004

Abstract

The total number of bacteria and culturable bacteria in Adélie penguin (*Pygoscelis adeliae)* guano was determined during 42 days of decomposition in a location adjacent to the rookery in Admiralty Bay, King George Island, Antarctica. Of the culturable bacteria, 72 randomly selected colonies were described using 49 morpho-physiological tests, 27 of which were subsequently considered significant in characterizing and differentiating the isolates. On the basis of the nucleotide sequence of a fragment of the 16S rRNA gene in each of 72 pure isolates, three major phylogenetic groups were identified, namely the *Moraxellaceae*/*Pseudomonadaceae* (29 isolates), the *Flavobacteriaceae* (14), and the *Micrococcaceae* (29). Grouping of the isolates on the basis of morpho-physiological tests (whether 49 or 27 parameters) showed similar results to those based on 16S rRNA gene sequences. Clusters were characterized by considerable intra-cluster variation in both 16S rRNA gene sequences and morpho-physiological responses. High diversity in abundance and morphometry of total bacterial communities during penguin guano decomposition was supported by image analysis of epifluorescence micrographs. The results indicate that the bacterial community in penguin guano is not only one of the richest in Antarctica, but is extremely diverse, both phylogenetically and morpho-physiologically.
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Keywords: Antarctica; Bacterial diversity; Penguin guano; 16S rDNA

1. Introduction

Microorganisms living under extreme environmental conditions are frequently used for studying ecophysiological and biochemical adaptations [1]. They also serve as sources of enzymes for biotechnological applications [2]. One might thus expect to find potentially interesting bacteria and fungi in Antarctic microbial communities, especially as these are still poorly characterized [3]. The aim of this study was to characterize one such community in Admiralty Bay at King George Island. Previous investigation indicated high diversity and abundance of the microbial community in the permanently snow- and ice-free regions of King George Island [4–8]. One of several sampling sites that contained diverse culturable bacteria was located close to a penguin

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doi:10.1016/j.femsec.2004.06.012

rookery [8]. The amount of penguin guano deposited by a breeding population on the shores of Admiralty Bay is about 6.4 tons (dry weight) per day [9], causing a significant impact on the local budgets of carbon, nitrogen, phosphorus and other minerals [10]. We thus considered it pertinent to determine bacterial diversity in penguin guano during its decomposition. Two approaches were used to identify and classify the cultivated bacteria, one based on morphological and physiological properties of purified strains and the second based on analysis of their 16S rRNA gene sequences. Cluster analyses based on the respective data sets showed similar groupings of isolates, indicating that conventional morpho-physiological tests such as those in an Appareils et Procédés d' Identification (API) micromethod may complement the 16S rDNA-based characterization of cultivated bacteria.

This study aimed at shedding light on the physiological and phylogenetic diversity of bacteria cultivated from decomposing penguin guano, and at designing a simple morpho-physiological assay to group these bacteria.

2. Material and methods

2.1. Sites and sampling

Adélie penguin (*Pygoscelis adeliae*) guano was collected from the rookery close to Rakusa Point, Admiralty Bay, King George Island, Antarctica (62°09′50″S, 58°28′W), between 29 December 1999 and 9 January 2000. Freshly deposited penguin guano was collected from the ground using a sterile plastic spatula and with no underlying soil into sterile 50 ml polypropylene tubes. Hundreds of samples (total >0.5 kg) were collected within few hours. Samples were immediately brought to the laboratory at the *Henryk Arctowski* station. In the laboratory, 60 subsamples of 5–7 g each were wrapped in sterile bolting-cloth (pore size 0.3 mm) bags. All transfers were conducted aseptically below 10 °C. Each wrapped sample was returned to the collection site and placed directly on the soil surface, and surrounded by a steel mesh enclosure to protect against trampling. Samples were left undisturbed for up to 42 days.

2.2. Bacterial isolation and characterization

On days, 0, 2, 6, 10 and then every 5 days up to 30 days (with a final collection on day 42), 6–7 bags were removed from the enclosure and returned to the laboratory. The number of bacterial colony forming units (CFU) in one gram (wet weight) of material aseptically collected from each bag was determined. Single subsamples of 1 g were homogenized in 10 ml phosphate-buffered saline (PBS, 0.15 M potassium phosphate, 0.85% NaCl, pH 7.0) [11] in a glass homogenizer. The homogenate was centrifuged for 7 min at 250*g*, and the supernatant poured into a sterile flask. The pellet was resuspended, and washed twice by centrifugation in sterile PBS. All fractions were pooled in a sterile flask and serially diluted (10^0–10^7) in PBS. Soil extract nutrient agar plates [12,8] were inoculated with 0.1 ml from each dilution in the series. The standard error (SE) in CFU counts was estimated from four replicates. After 15–25 days incubation at 10 °C, colonies were counted and CFU calculated per 1 gram of guano (dry weight). Randomly selected colonies were transferred to soil extract nutrient agar plates, which were incubated at 10 °C. Each was purified through repeated passages on the same medium. For 0, 2nd, 10th, 15th, 20th, 25th, 30th and 42nd experimental day 8–10 pure colonies were randomly selected (equal to ~10% of colonies) and subcultured, then characterized through morpho-physiological analysis and 16S rRNA gene analysis. To avoid errors due to overlapping and confluent growth, selections were made only from plates with less than 100 colonies.

Total bacterial counts (TC) in the same samples in which colony forming units were enumerated were determined following 4′6-diamidino-2-phenylindole (DAPI) staining on black Nuclepore polycarbonate 0.2 μm filters [13]. Samples were observed with a Nikon Eclipse E200 epifluorescence microscope, equipped with a UVA-2A Ex 330–380 filter, 100 W mercury lamp, 100X CFI 60 objective, and black and white COHU 4910 video camera (Mutech IV-410 card). A minimum of 400 bacterial cells per sample were counted automatically using an image analysis system (Lucia 4.60; Laboratory Imaging, Prague, CZ). The standard error (SE) was calculated for the average values of three measurements.

Diversity within the total bacterial population was calculated using a modified Shannon index [14], based on counts of specific morphological forms (cocci, rods, curved forms) evaluated in five cell volume classes; <0.1; 0.1–0.2; 0.2–0.5; 0.5–1.0, and >1.0 μm^3.

The ability of isolates to grow at 4, 22 and 32 °C, and colony pigmentation was determined on a nutrient medium containing 4% w/v NaCl. Motility, morphology and Gram-reaction were determined through microscopic observations. Other physiological features were determined in API 20NE and API ZYM assays (bioMérieux) according to the manufacturer's recommendations, with modifications to take into account the psychrophilic nature of the cultures.

Responses of isolates tested in API 20 NE were converted into numerical profiles according to the manufacturer's instructions, thus permitting comparison with those of type strains in the Analytical Profile Index [15]. Identification in the API system is based on comparing the observed profile to those in the database; a

T index also describes the strain's proximity to the most typical profile of each taxon.

The results of morpho-physiological analyses were encoded as binary data and clustered using the branch-and-bound Wagner parsimony method [16] provided by the PENNY program from the Phylip 3.6a package. The program ATV [17] was used to visualize the results of phylogenetic and cluster analyses.

2.3. DNA analysis

Genomic DNA from a single bacterial colony was extracted by phenol/chloroform extraction method. Cell pellets powdered in liquid nitrogen were digested with proteinase K (MBI) as recommended by manufacturer, at 56 °C for 3 h. Extracts were then digested with RNAse at 37 °C for 30 min. Following phenol extraction and isopropanol precipitation, DNA was collected by centrifugation (14k, 10 min, 4 °C), air dried, and resuspended in ultrapure water (30 μl). A fragment of the 16S rRNA gene from each genomic DNA sample was amplified using the forward primer L63f (5-CAGGCCTAACACATGCAAGTC-3′) and the reverse primer P1387 (5′-GGGCGGTGTGTACAAGGC-3′). An internal primer was used in sequencing reaction (INT1 5′-CAGCCGCCGCGGTAATAC-3′). PCR reactions were performed using 10 pmol each of L63f and P1387 in reaction mixtures containing 0.2 mM NTPs, 2.5 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (MBI) in the reaction buffer recommended by the manufacturer. Reactions were performed in a Perkin Elmer Cetus DNA Thermocycler. Following an initial denaturation step of 5 min at 94 °C, 35 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 45 s were performed. PCR products were visualized in an agarose gel and purified with a QIAquick PCR purification kit (Qiagen). Nucleotide sequences of the PCR products were determined using the DNA Sequencing Big Dye™ Terminator Cycle Sequencing Kit (PE Biosystems) and an ABI PRISM 377 DNA sequencer. All steps were performed according to the manufacturer's instructions.

2.4. Phylogenetic analysis and sequence accession numbers

DNA sequences were aligned in ClustalW [18] with sequences of the 22 closest matches from GenBank. Regions with excessive gaps were removed from the alignment prior to phylogenetic analysis using GeneDoc [19]. All phylogenetic analysis programs are in the Phylip 3.6a package [20]. 16S rRNA gene nucleotide sequences generated in this work were submitted to GenBank and given Accession Nos. AY377465 to AY377534.

3. Results and discussion

3.1. Total and culturable bacterial abundance and diversity

The abundance of the total bacterial fraction (total count, TC) in penguin guano during 42 days in situ incubation adjacent to a penguin rookery was determined by microscopy. Bacterial CFU were also enumerated, and described in terms of morpho-physiology and phylogenetic affiliation. Over the course of the experiment, the abundance of bacteria from guano, culturable on enrichment medium, increased from 0.28% to 91% of the total bacterial population determined by microscopy (Table 1). The very high CFU:TC ratios in the late phase of guano transformation contrasts with reports that cultivated bacteria represent less than 1% of the soil microbial community [21]. Indeed, total bacteria tended to reach their highest abundance 30 days after commencement of in situ incubation (Table 1). More substantial increases in CFU, however, were observed. Differences in the number of CFU and the ratio to TC during the experiment appear to be connected to changes in bacterial community composition. Extremely large fractions of the total bacterial community in fertile terrestrial habitats may in fact be culturable [22]. A high CFU:TC ratio has been described earlier in soil below the penguin rookery [8]. A significant increase in the di-

Table 1
Changes in total bacterial count (TC ± SE), colony forming bacteria (CFU ± SE) and Shannon index (Sh I) during penguin guano decomposition process

Day	TC × 10^{10}	CFU × 10^{10}	CFU:TC (%)	Sh I
0	1.01 ± 0.064	0.0028 ± 0.0005	0.28	0.72
2	3.25 ± 0.059	0.0093 ± 0.0013	0.29	0.79
6	1.36 ± 0.092	0.10 ± 0.011	7.35	0.84
10	5.89 ± 0.11	0.17 ± 0.024	2.89	0.92
15	15.8 ± 0.99	1.07 ± 0.062	6.77	0.92
20	16.6 ± 0.97	4.2 ± 1.08	25.30	0.94
25	34.7 ± 0.44	5.37 ± 0.26	15.48	0.94
30	38.0 ± 1.23	11.0 ± 1.59	28.95	0.92
42	21.9 ± 1.10	20.0 ± 1.92	91.32	0.90

versity index [14] was observed (Table 1). With the aid of nonlinear regression (Zdanowski et al., unpublished) we determined that morphological diversity and exposure time were significantly and positively correlated up to 14 days of exposure ($p = 0.02$). Changes in the distribution of bacterial cell volumes, morphologies and biomass are to be presented elsewhere. According to Bölter [23], however, morphological patterns within bacterial communities at different sites change as a function of organic matter and other physical and biological factors, and "become distinguishable like fingerprints by their distributions of length, volume or surface".

The unique nature of Adelie penguin guano is determined largely by the penguin's diet, which comprises almost exclusively (95.4%) of krill (*Euphausia superba*) [24,25]. The nature of colonizers of guano could be linked to krill-associated bacteria. Donachie and Zdanowski [26] presented evidence that bacterial growth occurs in the animal's stomach, that bacteria in the stomach of live Antarctic krill participate in the digestion of host dietary components, and that CFU in the stomach (10^8 per gram of stomach) averaged ~4% of the respective the direct count. We can expect that the microbial community of penguin guano is largely of marine origin, such as that associated with krill. According to Trivelpiece (personal communication) the retention time of food in the penguin gut is less than 24 h. Once deposited on land, however, penguin guano is ultimately transformed into material that forms specific ornithogenic soils [10,27,28], a process affected by complex biological and physical factors. Soil is generally considered a habitat containing the most diverse microbial communities [29]. Curtis et al. [30] estimated that one ton of suburban garden soil contains 4×10^6 different taxa, or twice that of the entire ocean. High diversity in both bacterial habitats and community composition is reported here. Some of the guano samples incubated at the edge of the rookery were occasionally flooded by rain or melting snow from adjacent soils [8], thus effectively introducing terrestrial bacteria to the communities already in the guano.

The dynamic nature of the bacterial community in decaying penguin guano is evidenced by the fact that CFU in guano increased from <1% to 90% of the total count as the guano decayed. As shown in our previous report [31], bacterial counts of all investigated groups increased. Within the total CFU population, copiotrophic bacteria (which have higher growth rates on rich complex media) were ca. two orders of magnitude more abundant than oligotrophic bacteria. Chitynolytic bacteria in guano were detected only late in the incubation; by 42 days, >40% of the initial chitin content remained (Zdanowski et al., unpublished). Our studies on bacterial decomposition of penguin guano in the region [31]; (Zdanowski et al., unpublished) have shown the most important factors influencing decomposition and diversification of the bacterial community to be temperature and precipitation. Throughout the experiment described here, diurnal surface temperature ranged from −2.5 to +3.0 °C, and the maximum ranged from 3.3 to 11 °C; precipitation ranged from 0 to 20 mm m^{-2}. Another factor accompanied by diversification of the bacterial community is a change in pH. That in the surface soil throughout the penguin rookery, including at the experimental site, was alkaline (pH 8.8–9.3), but within penguin guano itself the pH increased rapidly from 5.9 in fresh guano, to 9.1 after 21 days. Such increasing alkalinity with time is likely due to the release of ammonia during mineralization of amino acids and uric acid [31].

3.2. Phenotypic tree

We determined phenotypic characteristics of 72 bacterial strains randomly selected from nutrient media plates inoculated with penguin guano. However, on some plates many colonies were overgrowing others and these could not be separated. All isolates grew at 4 °C on nutrient agar, and some also grew at 22 °C. The latter can be classified as psychrotrophs or psychrotolerant [32], while those not growing at 22 °C are psychrophiles. Only a handful of isolates grew at 32 °C.

Isolates were grouped on the basis of their responses in 49 morpho-physiological tests (Fig. 1(a)). The first character in the isolate code refers to the consecutive number in which an isolate was selected, and the next figure (in bracket) refers to the experimental day on which the guano was collected.

Four groups comprising 10–28 strains and two unclustered isolates 12-(2) and 14-(2) were distinguished. Cluster I was comprised of 28 isolates, 14 of which were related to species within the *Moraxellaceae*/*Pseudomonadaceae* or *Pasteurellaceae* according to the API 20NE database. Cluster II contained 10 isolates, two of which were related to species within the *Flavobacteriaceae*. API 20NE numerical profiles of isolates in clusters III and IV had no matches in the bioMéreiux database. Bacteria in the collection were morpho-physiologically diverse. Among 72 randomly selected strains, only fourteen had an identical or very similar neighbor within the collection. Such high diversity was apparent through each of the API 20NE, API ZYM, and conventional tests. It should be noted, however, that ten strains in cluster I shared the same numerical profile in API 20NE, corresponding to a *Moraxella* spp., although subsequent data from API ZYM and other morpho-physiological tests showed these strains could be differentiated.

3.3. Effective discrimination by morpho-physiological tests

We examined which of the 49 morpho-physiological tests were the most effective in differentiating and grouping our isolates and which of them could be omitted in

such analysis (Fig. 2). The data were presented through cluster analysis organizing the data into a meaningful structure [33]. During this procedure, the number of tests was reduced step by step, depending on the Manhattan distances among the corresponding rows of binary matrices, the representation of data. We initially eliminated tests in which all responses were 100% positive or 100% negative, and then gradually eliminated more of those with the lowest number of differentiating responses. PENNY, in the PHYLIP package, was used since this is appropriate for analysis of binary data [34]. Twenty seven tests were ultimately selected as meaningful, comprising five conventional tests, twelve API 20NE tests and nine API ZYM tests. It was the smallest number of tests that determined almost the same topology of phenotypic tree as the topology of the tree based on 49 tests. Further elimination generated scattering of the *Moraxellaceae* and *Firmicutes* (data not shown).

3.4. Phylogenetic trees

We prepared an alignment of 16S rRNA gene sequences from 70 isolates and 22 published sequences covering 526 nucleotides, after removal of regions containing excessive gaps. A distance matrix was calculated in DNADIST using the F84 nucleotide substitution model. Other models such as the LogDet distance or the Jukes-Cantor model gave similar results (data not shown). A phylogenetic tree was constructed using the neighbor-joining algorithm (NEIGHBOR program) (Fig. 1).

On the basis of nucleotide sequences described above, our strains grouped into three major clusters. The first comprised 28 strains and seven sequences from the public domain all affiliated with *Moraxellaceae*/*Pseudomonadaceae* in the γ-proteobacteria. Four isolates (92, 94, 97 and 114), however, were less closely related to this cluster and affiliated more with *Oleispira antarctica* [γ–proteobacteria, Oceanospirillaceae] and an uncultured Antarctic soil bacterium clone (Fig. 2). The second cluster comprised strains and sequences from the CFB group, while the third comprised members of the phyla *Actinobacteria* and *Firmicutes*. One isolate (250) in the latter group is related to *Planococcus southpolaris*.

Bootstrapping with 500 re-samplings was used to estimate the robustness of the tree. The resulting consensus tree showed a topology similar to that based on phenotype (Fig. 2), although the bootstrap values were low (<20% for most nodes). This is to be expected in a global tree containing both distant and closely related sequences. The trichotomy grouping was confirmed by independent phylogenetic methods, namely in the maximum parsimony (program DNAPARS) and the maximum likelihood trees (program DNAML) (data not shown).

3.5. Phylogenetic relations within subgroups

In order to investigate the three main clusters more closely, a phylogenetic analysis was repeated for each separately, following the procedure described above for the global tree. As the sequences within each group aligned better than in the global tree, longer alignments were used (630, 606 and 644 nucleotides for the first, second and third groups, respectively) (Fig. 3).

Sequences in the first cluster are related to the *Moraxellaceae*/*Pseudomonadaceae* within the γ–proteobacteria, and comprise two subgroups (Fig. 3(a)). One group containing four isolates (92, 94, 97 and 114) plus *Oleispira antarctica* and an uncultured Antarctic soil bacterium clone AF447179. The second group consists of many closely related sequences, but the data is not sufficient to infer a robust classification. The second cluster contains sequences related to the *Flavobacteriaceae* (Fig. 3(b)). Within the first subgroup are three *Chryseobacterium* sequences and *Riemerella columbina*, which cluster with isolate 26, while another eight isolates affiliate with *Chryseobacterium* sp. p20H. The next subgroup locates isolate 5 with two *Gelidibacter* sequences, and isolates 98 and 249 with a *Flavobacterium* sp. The third cluster contains sequences closely related to the *Arthrobacter* and one from the *Firmicutes* (Fig. 3(c)).

The phylogenetic analyses show also a high diversity within our bacterial collection. Among 72 randomly selected bacterial strains, only four isolates were identical or very similar to one another. Three isolates (75, 105 and 108) were thus eliminated from the phylogenetic tree since they were represented by other isolates (73, 71 and 83, respectively).

3.6. Comparison between groups based on phenotypic responses and on DNA sequences

We tested if the trees based on morpho-physiological and molecular data were similar in terms of how their component isolates grouped. A tree based on the responses to 49 morpho-physiological tests was used as part of this comparison. It is clear that trees based on phenotypic or molecular data were structurally similar, with the same isolates grouping together in each (Figs. 1 and 2).

Of 28 strains considered to be *Moraxellaceae*/*Pseudomonaceae* based on the DNA sequences (Fig. 3), as many as 25 clustered similarly on the basis of phenotypic responses (Fig. 1). Also, of 13 strains considered *Flavobacteriaceae*-like, nine strains based on molecular data, were clustered in a similar way in the phenotypic tree (Fig. 1). Twenty five strains of the group of 28 strains, identified as *Actinobacteria* and *Firmicutes* occupied similar positions in both trees (Figs. 1 and 2).

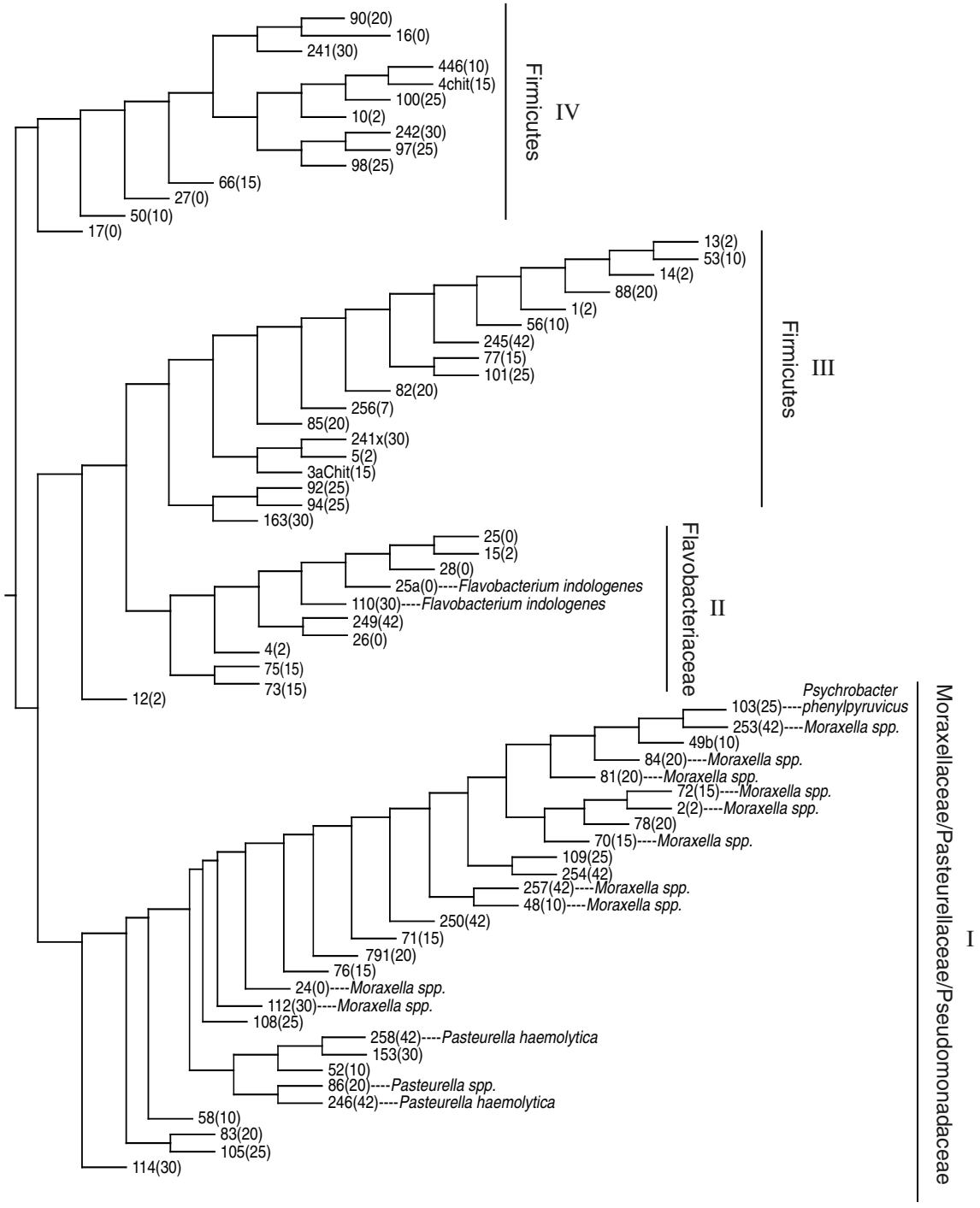

Fig. 1. (a) Hierarchical classification of 72 purified CFU isolates from penguin guano, based on their responses in 49 morpho-physiological tests. Isolate labelling is described in the text. The following tests were conducted: Ability to grow at 4, 22, and 32 °C, and to grow on nutrient agar containing 4% w/v NaCl. Morphological analyses comprised counts of pigmented colonies (PIGM), three microscopic tests – motility (MOT), morphology (ROD) and Gram reaction (GM). The API 20NE system allows 10 biochemical tests: reduction of nitrate to nitrite (NO_2), and to nitrogen (N_2); indole production (TRP); fermentation of glucose (GLU-F); arginine dihydrolase (ADH); urease (URE); β-glucosidase (ESC); gelatin hydrolysis (GEL); β-galactosidase (PNPG); cytochrome oxidase (OX); and 12 tests for assimilation of carbohydrates as sole carbon sources; glucose (GLU); arabinose (ARA), mannose (MNE); mannitol (MAN); *N*-acetyl-glucosamine (NAG); maltose (MAL); gluconate (GNT); caprate (CAP); adipate (ADI); malate (MLT); citrate (CIT); phenyl-acetate (PAC). The API ZYM system tests for the presence of 19 constitutive enzymes: alkaline phosphatase (Bph); esterase – C_4 (Est); esterase lipase – C_8 (Esl); lipase – C_{14} (Lip); leucine arylamidase (Leu); valine arylamidase (Val); cystine arylamidase (Cys); trypsin (Try); chymotryp-sin (Chy); acid phosphatase (Aph); naphthol-AS-BI-phosphohydrolase (Nap); –galactosidase (αGa); β-galactosidase (βGa); β-glucouronidase (Gl), α-glucosidase (αGs); β-glucosidase (βGs); *N*-acetyl-β-glucosaminidase (Nac); α-mannosidase (αMa); α-fucosidase (αFu).

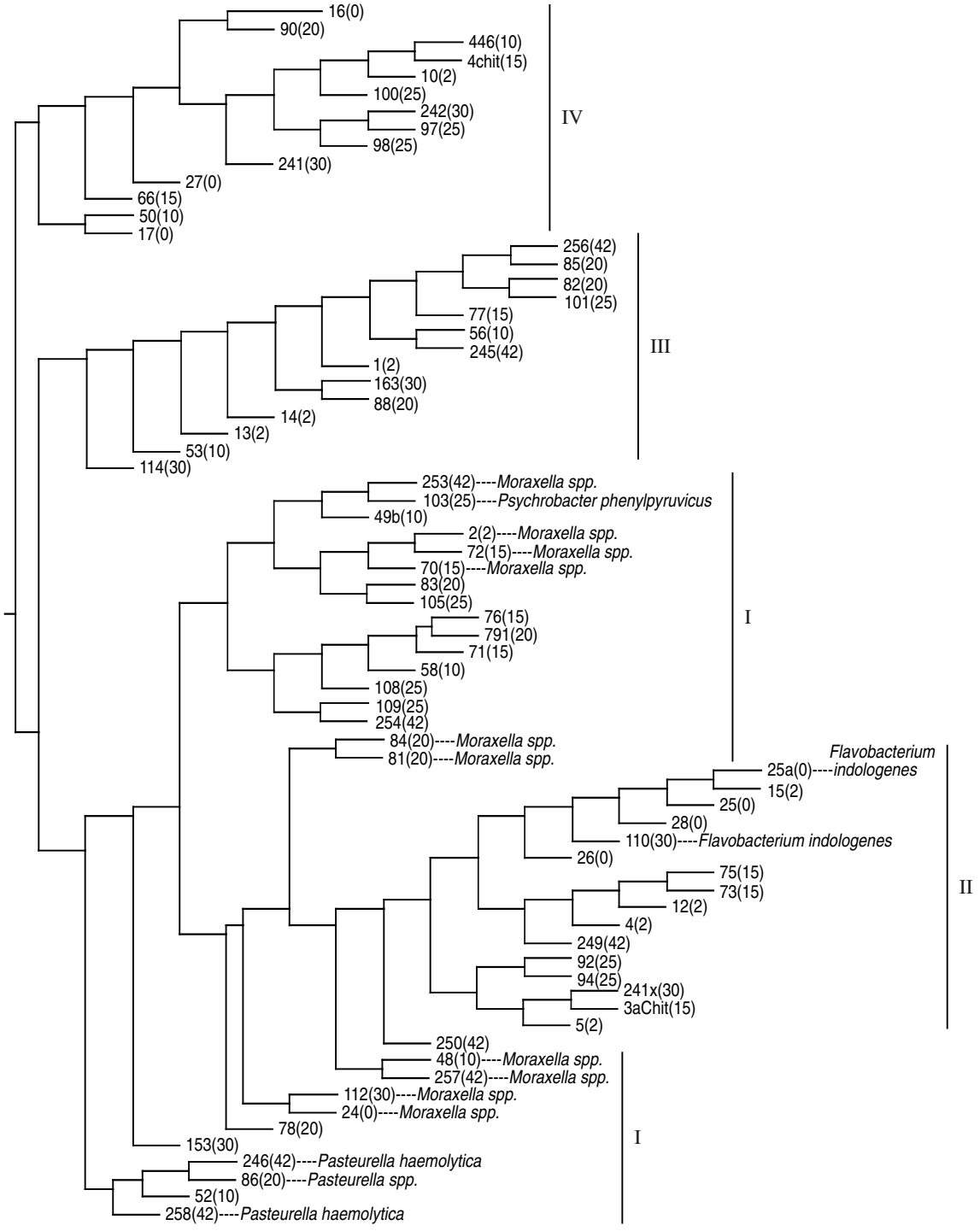

Fig. 1. (b) Hierarchical classification of responses in 27 morpho-physiological tests of 72 pure CFU isolates from penguin guano. Isolate labelling is described in the text. The tree is based on responses in the following tests: ability to grow 22 °C, and ability to grow on nutrient agar containing 4% w/v NaCl, PIGM, ROD, MOT, NO_2, TRP, URE, ESC, GEL, PNPG, OX, MAN, MAL, GNT, MLT, CIT, PAC, Bph, Est, Esl, Val, Chy, Aph, Nap, βGa, αGs (see (a) for abbreviations).

3.7. Phylogenetic diversity of bacteria cultivated from penguin guano

The maritime Antarctic, with its complex yet patchy soil ecosystems, provides microenvironment conducive to high bacterial abundance and diversity [8]. In relatively small areas free of ice at the marine and terrestrial frontier, marine bacteria are transported to the land in aerosols and through avian influences, while those of terrestrial origin are transported in run-off waters and carried into the sea on winds and by birds. Other Antarctic habitats further south may host less diverse com-

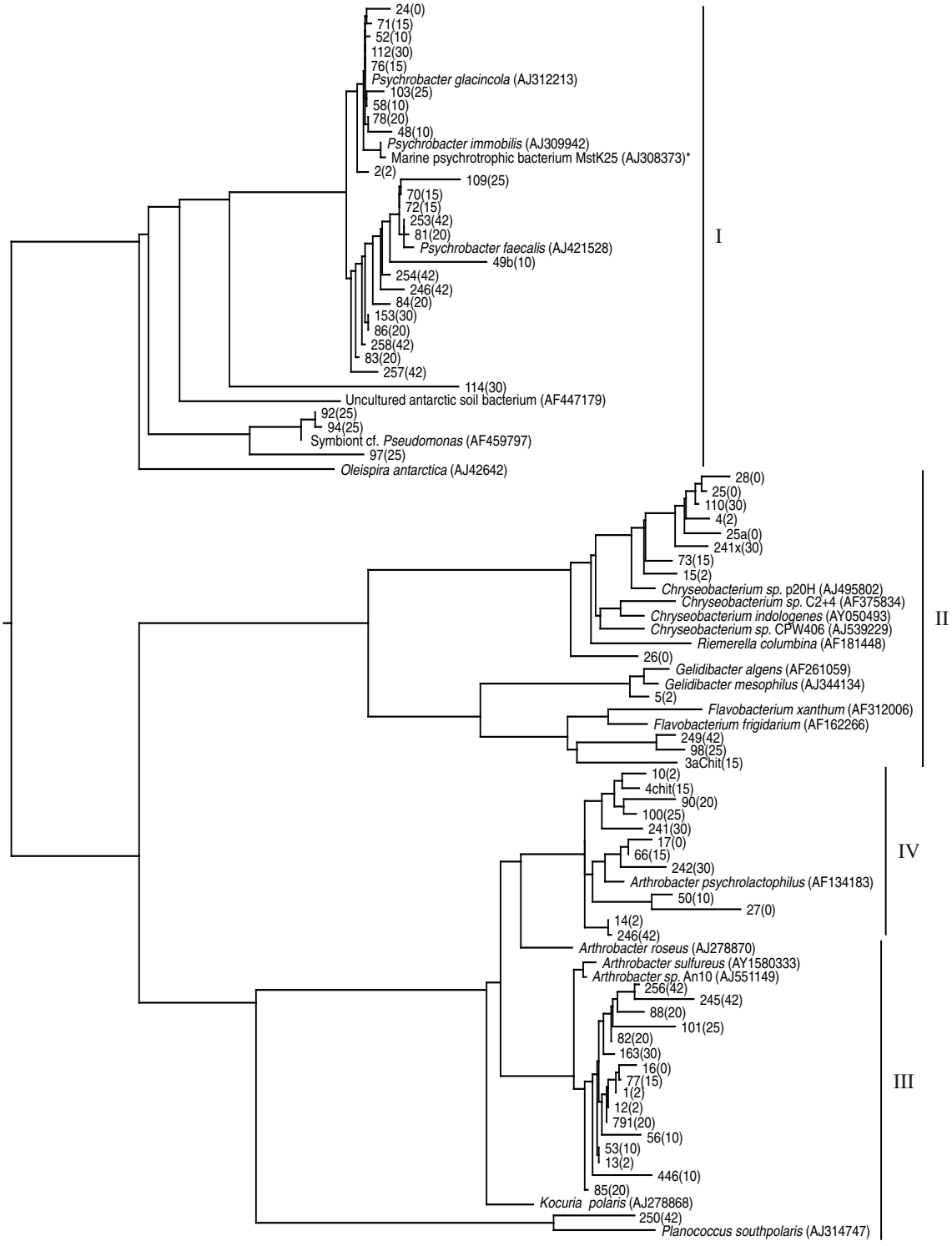

Fig. 2. Neighbour joining phylogeny of 16S rDNA sequences from 70 Antarctic bacterial isolates cultivated from penguin guano. Related sequences from the public domain are included. See Fig. 1 for description of isolate numbering. *, closely related to *Psychobacter* sp.

munities because of isolation and climatic pressure [35]. Most Antarctic microorganisms appear to belong to a small number of cosmopolitan taxa [3]. It should be borne in mind that under the rather unique conditions of isolation and climate in Antarctica, many adaptations or even speciation events could have occurred.

Representatives of one of the largest groups identified in this study, the *Moraxellaceae/Pseudomonaceae*

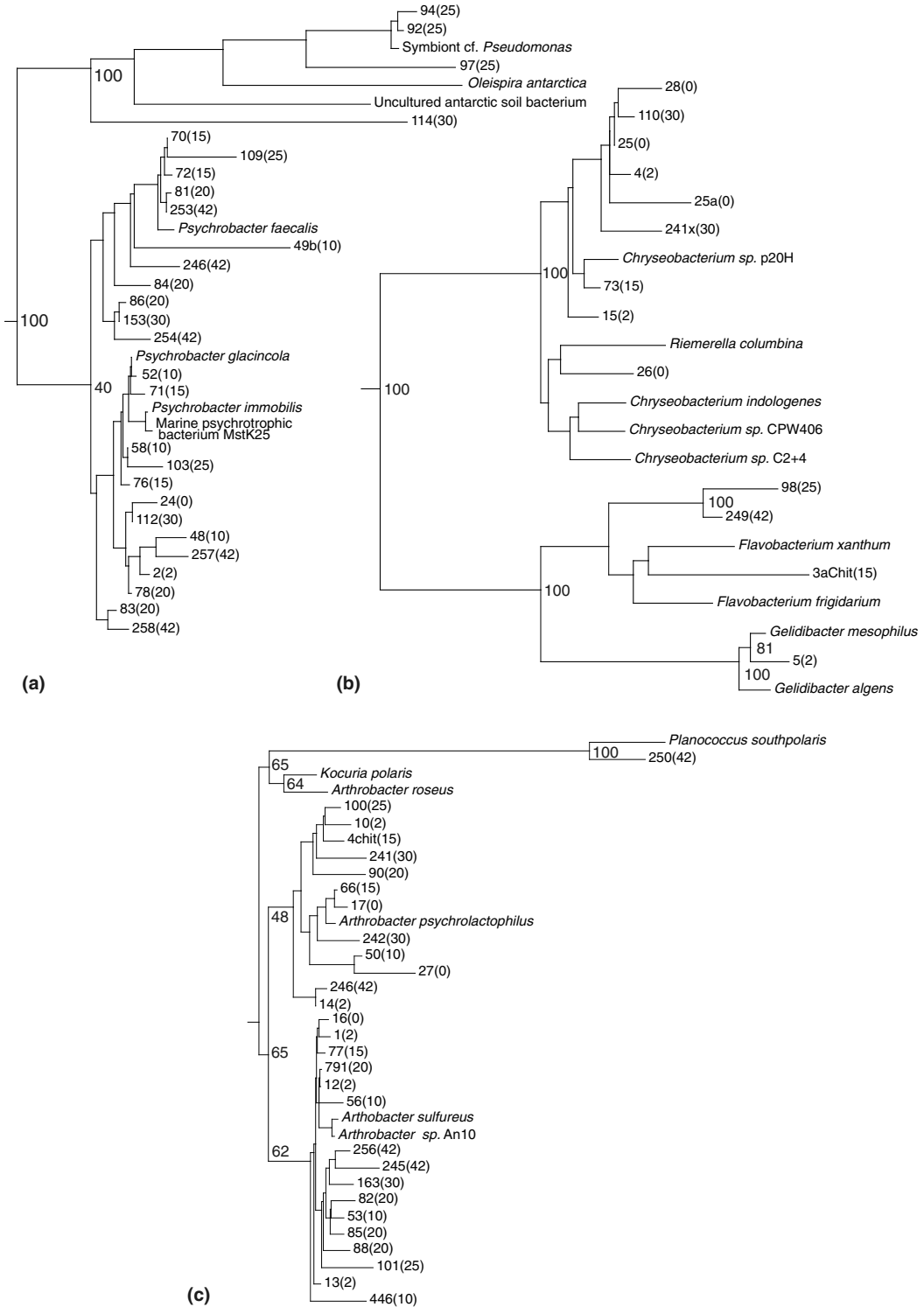

Fig. 3. Phylogenies of three major clusters of cultivated Antarctic bacteria; *Moraxellaceae*-like (a), *Flavobacteriaceae*-like (b) and *Firmicutes*-like (c), constructed by neighbor-joining based on a sequence distance matrix for the 16S rDNA gene. Percentage bootstrap values are shown at selected nodes.

Pasteurellaceae (MPP) within the γ–proteobacteria were scarce during the initial six days of the experiment, but became prominent in the later phase of guano decomposition. This might indicate a terrestrial origin of these non-pigmented bacteria migrating from soil to guano. This would agree with our previous observations [8] showing that bacteria of this group are prominent in different soils in the vicinity of a penguin

rookery. Their somewhat cosmopolitan nature is confirmed by their being the most frequently isolated bacteria from both uncontaminated soil at Mawson Base (East Antarctica) [36] and from sea ice [37]. Note, however, that our present studies revealed a number of morpho-physiological differences among these isolates (cf. Fig. 1). In contrast, members of the *Flavobacteriaceae* were prominent in the early stages of guano decomposition, inferring a marine origin, although their inability to grow in the presence of 4% (w/v) NaCl might make this hypothesis controversial. It is to be noted that these bacteria were not detected in our previous studies of Antarctic soil bacteria [8].

The largest group of pigmented bacteria comprised two subgroups related to the *Arthrobacter* (*Micrococcaceae*) and to a *Planococcus* sp. These genera are in fact considered cosmopolitan in soils. Indeed, the *Arthrobacter* may be numerically dominant among bacteria cultivated from soils as diverse as those in paddy fields and in Antarctica, a fact that may be attributed to their metabolic diversity [38]. Interestingly, increasingly many bacteria cultivated from environmental samples are phylogenetically unique.

To conclude, we compared how grouping of randomly selected isolates on the basis of their responses in morpho-physiological tests would compare with groups derived from their 16S rRNA gene nucleotide sequences. Our results indicate that these approaches agree in the composition of the respective groups, and that a selected range of morpho-physiological tests can rapidly group strains to the same standard as tests based on sequences of 16S rRNA genes. This approach could be considered as a tool in the classification of bacteria cultivated from environmental samples. Moreover, our data show that bacterial community of the penguin guano is highly diverse. Penguin guano represents material principally of marine origin but whose decomposition can proceed rapidly on land.

Acknowledgements

This study was supported by a grant from the Polish Committee for Scientific Research (KBN) No. 6 PO4F 016 18. We thank Prof. Stanislaw Rakusa-Suszczewski for excellent working facilities at *Henryk Arctowski* Station and at the Department of Antarctic Biology, Polish Academy of Sciences. We are very grateful to S. Donachie (University of Hawaii) for comments and English editing during preparation of this paper.

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