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ORIGINAL PAPER

Sensitive change of iso-branched fatty acid (iso-15:0) in *Bacillus pumilus* PAMC 23174 in response to environmental changes

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Abstract In this study, the environmental adaptive metabolic processes were investigated using a psychrotrophic polar bacterium *Bacillus pumilus* PAMC 23174 in response to various temperatures and nutrients, especially in regard to the synthesis of fatty acids. Fatty acid methyl ester analysis was performed using gas chromatography–mass spectrometry and we found that a sensitive changes in iso-branched fatty acid (iso-15:0) synthesis occurred when adjusting the nutritional ratio of branched chain fatty acids (anteiso/iso) with different temperatures, resulting in a change in the balance of anteiso- and iso-form fatty acids. We also observed that this Arctic bacterium preferred amino acid leucine for the synthesis of fatty acids. The increased and decreased synthesis of iso-form fatty acids in response to different temperatures and leucine preference, changes the fatty acid ratio in bacteria, which further affects the membrane fluidity and it is also directly correlated with survival of bacteria in an extreme environment. Hence, this study suggests that *B. pumilus* PAMC 23174 is a potential model organism for the analysis of the unique ecological adaptations of polar bacteria in changing and the extreme environments.

Keywords Polar bacteria · *Bacillus pumilus* · Climate change · Adaptation · FAME · GC–MS

Introduction

Most indigenous bacterial populations have their own survival mechanisms and respond to environmental changes or conditions such as fluctuations in temperature and external osmolarity [1]. These adaptive mechanisms are mainly evolved for the survival of microorganisms in extreme environments and these adaptive processes also controls the membrane fluidity according to temperature fluctuations. This adaptation results the transition of liquid crystalline into a gel-like phase state of the lipid bilayer under increased temperature conditions. At present, there are three strategies to change the membrane fluidity by modifying the fatty acid composition in bacteria. The first process which balancing the amount of iso- and anteiso-form odd-numbered fatty acids, the second is conversion of saturated fatty acids into unsaturated fatty acids by the action of desaturase, and the third is the production of shorter fatty acids within the bacteria [2]. Currently, most of the existing literatures focus on mesophilic bacteria like *Bacillus subtilis*, but there are only few reports on polar bacteria that prefer to live in psychrophilic/psychrotrophic nature and that can survive in extreme Arctic/Antarctic regions [3, 4]. Thus, we intend to investigate the polar bacterial lipid profile in order to study about the adaptation of these polar bacteria in extreme conditions, and to provide more evidence for the understanding of the changes that occur in polar bacteria. This

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understanding is also an important thing in the study of mesophiles that may require adaptation to colder weather in the future due to climate change. In this study, we investigated a strain of polar bacteria, *B. pumilus*, which produces simpler type of fatty acids than other *Bacillus* species, and we monitored the changes in the fatty acid profile in this bacterium in response to different culture conditions. Based on this study, we found that the simple profile of the fatty acids was advantageous in monitoring changes in lipid production in polar bacteria. The results of our experiments showed that strain *B. pumilus* utilize iso- and anteiso-form odd-numbered fatty acids synthesis as a major mechanism to adapt to the changing environment, similar to other *Bacillus* sp. [5, 6]. However, in contrast with other studies, we found that there is an adjustment related with sensitive changes of the iso-15:0 fatty acids to control the ratio of anteiso/iso-form fatty acids synthesis. This report highlights the changes in fatty acid production under different temperature conditions in a bacterium isolated from the Arctic region.

Materials and methods

Bacterial strains and growth conditions

Arctic strain *B. pumilus* PAMC 23174 and *Bacillus thuringiensis* PAMC 23133 were obtained from the Polar and Alpine Microbial Collection (PAMC), Korea Polar Research Institute (KOPRI), and *Bacillus cereus* KCTC 3624 and *B. thuringiensis* KCTC 3452 were obtained from the Korean Collection for Type Cultures (KCTC). All *Bacillus* strains were grown in Tryptic Soy broth (TSB), Luria–Bertani (LB) and M9 minimal media with yeast extract at respective temperatures of 20, 30, and 37 °C for 24 h under shaking (200 rpm).

Lipid extraction from cells and fatty acid methyl ester (FAME) derivatization

10 mg of lyophilized *B. pumilus* was used as biomass, 0.9 mL of each organic solvent was added into the biomass for lipid extraction from the cells, and then the mixture was

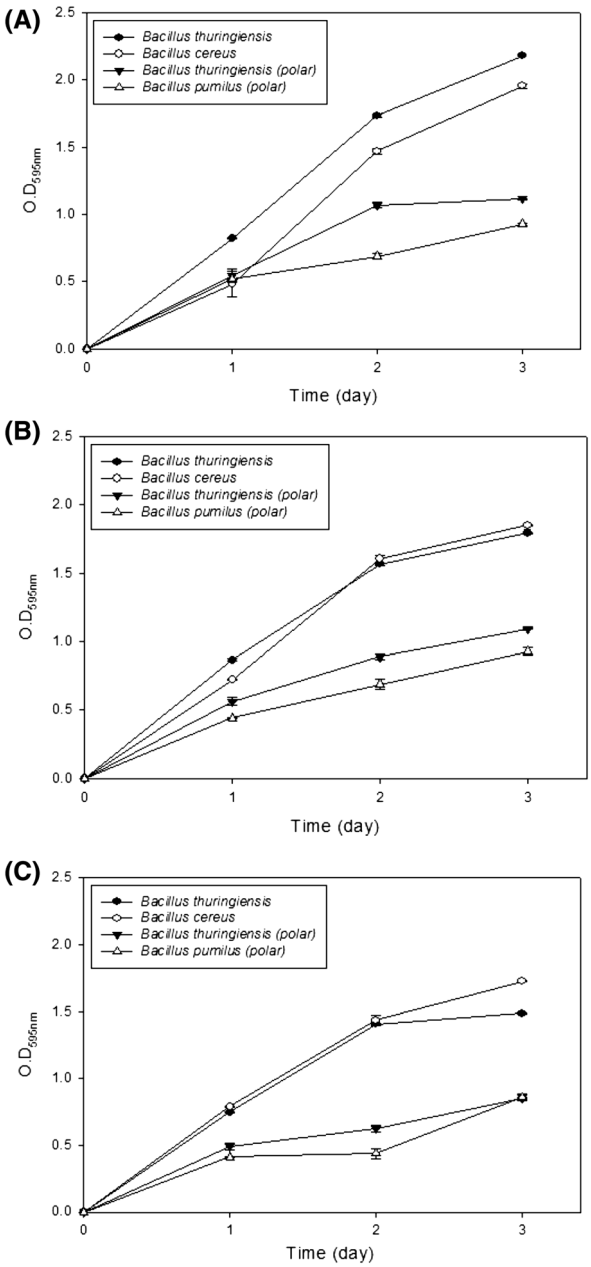

Fig. 1 The growth of four *Bacillus* sp. at three different temperatures over 3 days. Temperatures are **a** 20 °C, **b** 30 °C and **c** 37 °C each

Table 1 Environmental features of Arctic strains *Bacillus pumilus* and *Bacillus thuringiensis*

PAMC 23174		PAMC 23133	
Locality	Kara Sea (Arctic Ocean)	Locality	Kara Sea (Arctic Ocean)
Latitude (start)	(N) 73.80777	Latitude (start)	(N) 73.00188
Longitude (start)	(E) 64.38692	Longitude (start)	(E) 63.51984
Depth	0.05–0.1	Depth	−0.05
Habitat	Sediment (marine)	Habitat	Sediment (marine)
Temperature (°C)	10–37	Temperature (°C)	15–37
pH	6–9	pH	6–10

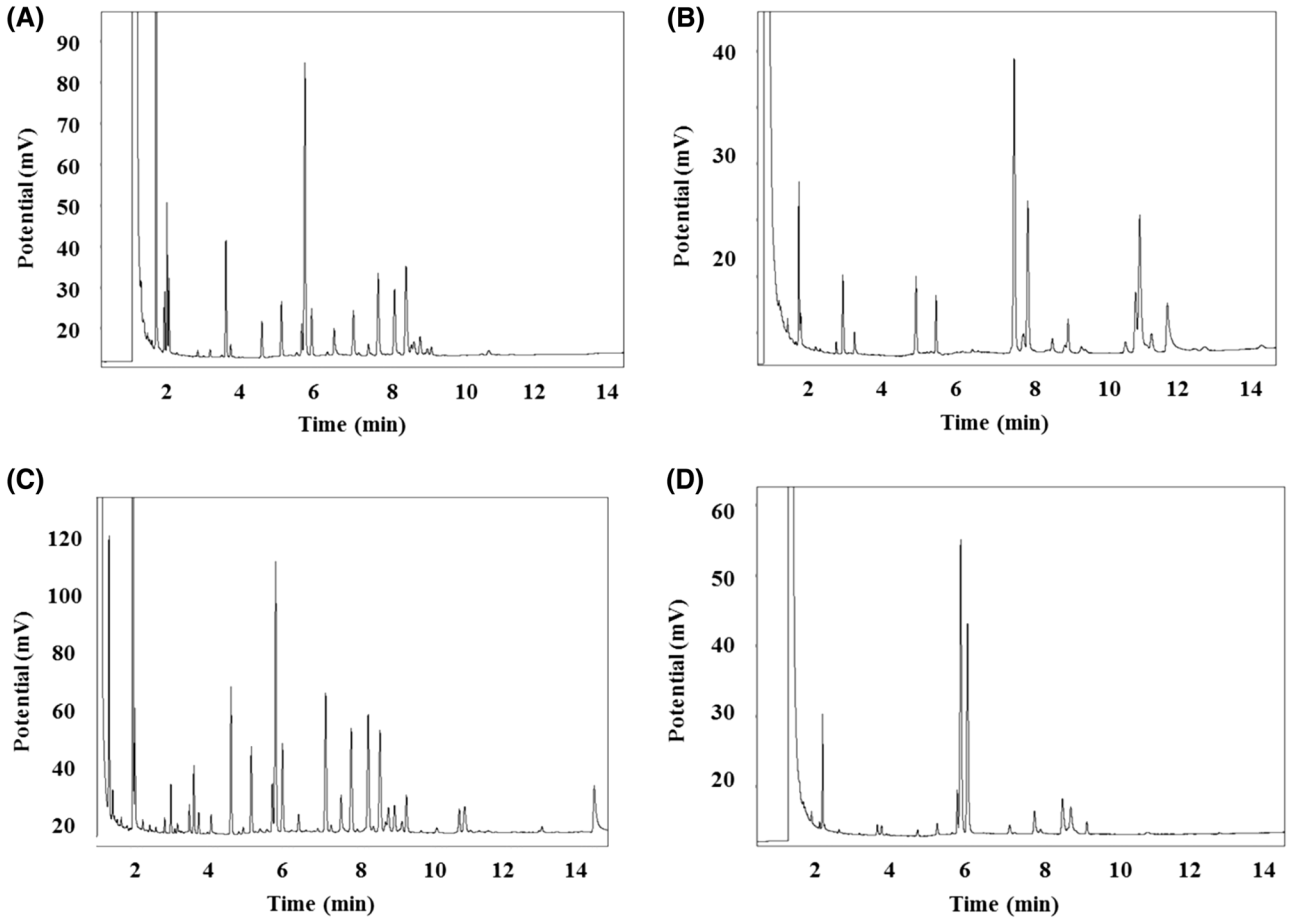

Fig. 2 Whole cell fatty acid analysis of four *Bacillus* sp. species by GC-FID. **a** *Bacillus thuringiensis* (KCTC 3452), **b** *Bacillus cereus* (KCTC 3624), **c** *Bacillus thuringiensis* (PAMC 23133), **d** *Bacillus pumilus* (PAMC 23174). The cells were cultured at 30 °C for 24 h

vortexed and activated at 37 °C for 1 h. The mixture was then centrifuged at 1500 rpm for 5 min and 0.45 mL of the liquid phase was transferred to glass vials, and the sample was evaporated and re-treated with 1 mL of chloroform for esterification. The quantification and composition of the whole cell fatty acids including membrane fatty acids were determined by gas chromatography (GC) according to literatures with certain modifications [7, 8]. To prepare methylated fatty acids, 1 mL $CHCl_3$ and 1 mL CH_3OH/H_2SO_4 (85:15, v/v) were added into the samples, then incubated at 100 °C for 2 h and further cooled on ice for 5 min. After adding 1 mL of cold distilled water, the samples were vortexed thoroughly for 1 min, and then centrifuged at 2000 rpm. The organic phase was then extracted and transferred into clean borosilicate glass tubes containing 1 mg of Na_2SO_4.

Analytical techniques

The resulting samples were analyzed by GC–MS (Younglin, Korea) equipped with a fused silica capillary column (Agilent HP-FFAP, 30 m × 0.32 mm, i.d. 0.25 μm film) containing a linear temperature gradient (for fatty acid: at 140 °C 1 min, 5 °C/min to 235 °C, hold for 5 min). The injector port temperature was set at 210 °C. Mass spectra were obtained by electron impact ionization at 70 eV, and scan spectra were obtained within the range of 100–600 m/z. The selected ion mode (SIM) was used for the detection and fragmentation analysis of major products.

Providing precursors for the synthesis branched-form fatty acids and cold shock treatment

Leucine (Leu), isoleucine (Ile), and valine (Val) (Sigma–Aldrich) were prepared with 100 mM stocks dissolved in distilled water (DW). These three amino acids were provided to the Luria–Berteni (LB) media as precursors of branched chain fatty acids, each at 1 mM concentration. The seed culture was prepared and the 2 % of the preculture was inoculated into LB media. The cold shock experiment was performed by freezing of bacterial culture at −80 °C for 20 h, and then 4 h thawing at room temperature (RT). All the experiments were conducted with duplicates.

Results and discussion

Growth of *Bacillus* sp. at different temperatures and identification of fatty acids by GC–MS

The four *Bacillus* spp. were closely related and the only difference is two of them were isolated from polar environment. Two strains such as *B. pumilus* PAMC 23174 and *B. thuringiensis* PAMC 23133 were from polar environment but exhibit the psychrotrophic (psychrotolerant) nature of growth, which means they grow well at above 20 °C. The strains *B. pumilus* PAMC 23174 and *B. thuringiensis* PAMC 23133 grew at low temperatures but it is a tolerant activity. The environmental features of Arctic bacterial strains are represented in Table 1. Our main intention is to know, how the nutritional fluctuation and environmental temperature affects the survival of these psychrotolerant bacteria in the polar region and their consequences in fatty acid synthesis. Hence, we had chosen two psychrotrophic bacteria along with two mesophilic strains (KCTC 3624 and KCTC 3452) to reveal and compare their FAME pattern in response to temperature and nutritional parameters. The polar bacteria exhibited slow growth at all temperatures (20, 30, and 37 °C) (Fig. 1) and its due to the slow psychrotrophic metabolic activity of polar bacteria [9]. After lipid analysis, strain *B. pumilus* PAMC 23174 has revealed a very simple profile of fatty acids while comparing with other *Bacillus* species and exhibit only a few types of fatty acid moieties that possess 14–18 carbons (Fig. 2). Five major fatty acids produced by *B. pumilus* were identified by GC–MS as 13-methyl tetradecanoate (iso-15:0), 12-methyl tetradecanoate (anteiso-15:0),

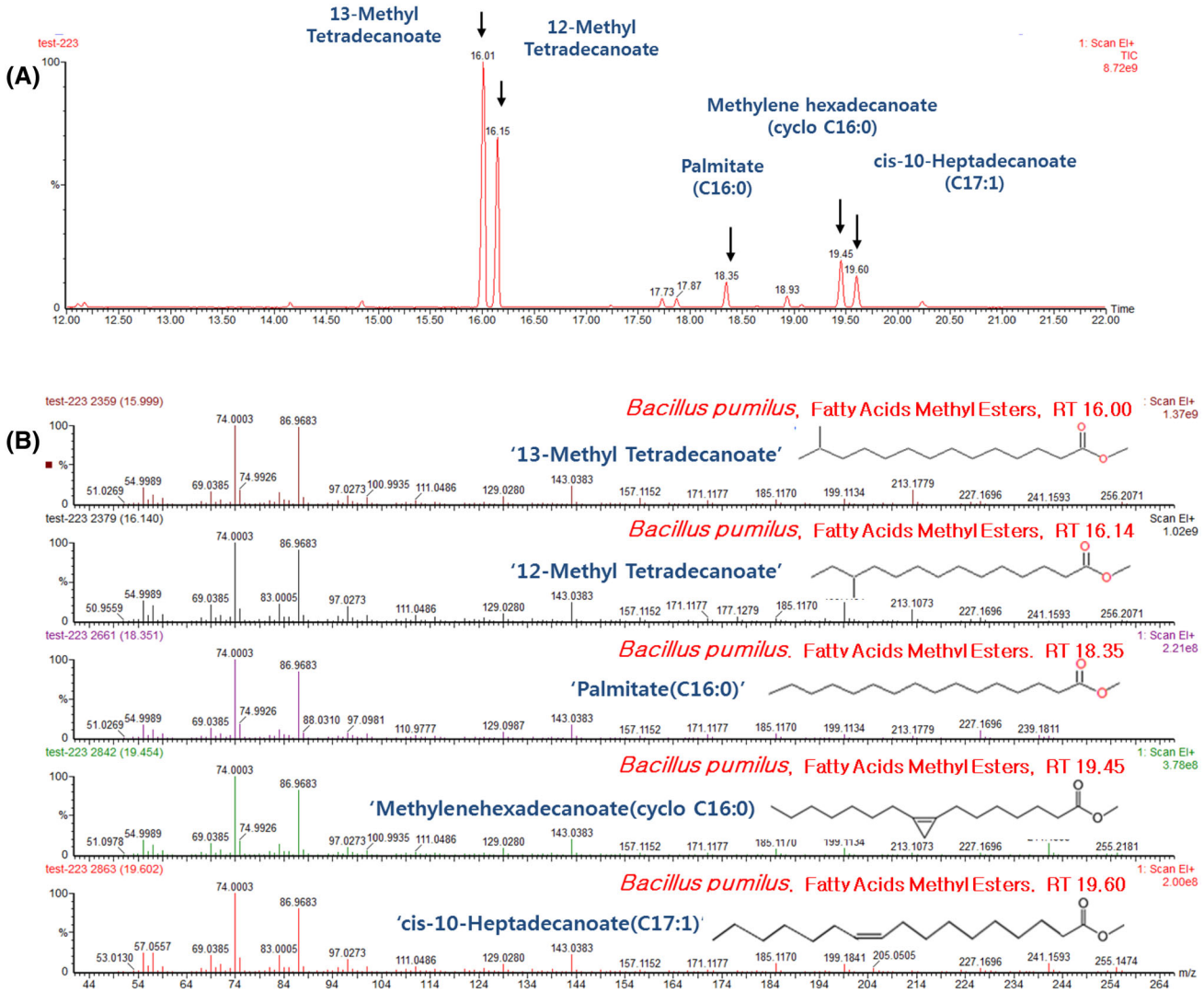

Fig. 3 Identification of major fatty acids in *Bacillus pumilus* PAMC 23174 by GC–MS

hexadecanoate (16:0), methylene hexadecanoate (cyclo 16:0), and cis-10-heptadecanoate (17:1) (Fig. 3). The relative portions of each main fatty acid such as iso-15:0, anteiso- 15:0 and 16:0, and cy-16:0 and 17:1, were calculated as 100 (the largest peak), 62.2, 9.0, 12.5, and 12.5, respectively.

Lipid inhibitor treatment and effects on the FMAE profile in *Bacillus pumilus*

The polar bacterium *B. pumilus* was cultured at various temperatures with the strain showing maximum growth at 30 °C. Although it was isolated from a polar region but it has the ability to grow at mesophilic temperatures and can be psychrotrophic (psychrotolerant) (Fig. 4a). These kinds of phenomena were also widely found in other bacteria that were isolated from psychrophilic environments [9, 10]. When its lipid production was monitored, the occupancy of anteiso-15:0 was clearly highest at 15 °C, but in contrast iso-15:0 was highest at 30 °C (Fig. 4b). The obtained results were identical with previous studies on the synthesis of branched form fatty acids in *B. subtilis* [11, 12] and it was commonly assumed that anteiso-form isomers are responsible for the increased membrane flexibility in the bacterial cells at low temperatures [5, 13]. In relation to the control of the overall ratio of anteiso- and iso-form fatty acids, iso-15:0 is more sensitive to temperature changes since the variation in quantity was larger than anteiso-15:0. Although, we do not know the exact reasons behind these changes and it is noteworthy to study about the increase of anteiso:iso fatty acid ratio in *B. subtilis*. Also, this strain could not efficiently shorten the fatty acid chain length at low temperatures and the anteiso-15:0 content was significantly lesser (45 %) than the well-known model strain *L. monocytogenes*. A lower anteiso-15:0 content might be one of the reasons for the inability of *B. subtilis* to grow at refrigeration temperatures. Likewise, *B. pumilus* also act like psychrotrophic instead of psychrophilic and also its leucine preference would affect the membrane fluidity (relatively less) by synthesis of iso-15:0. Considering other major researches on *B. pumilus* which are focused on the production of psychrophilic bacterial lipases but our study highlights the primary role of the fatty acid anteiso-15:0 in membrane fluidity and our analysis also provide clues about the bacterial responses against to temperatures to protect themselves.

To examine and analyze the sensitive change of iso-15:0, orlistat, an inhibitor of fatty acid synthetase (FAS) was supplemented into the medium to inhibit fatty acid synthesis, which results the significant reduction of iso-15:0 synthesis and further decrease the amount of total fatty acid synthesis. The fatty acid profile details in

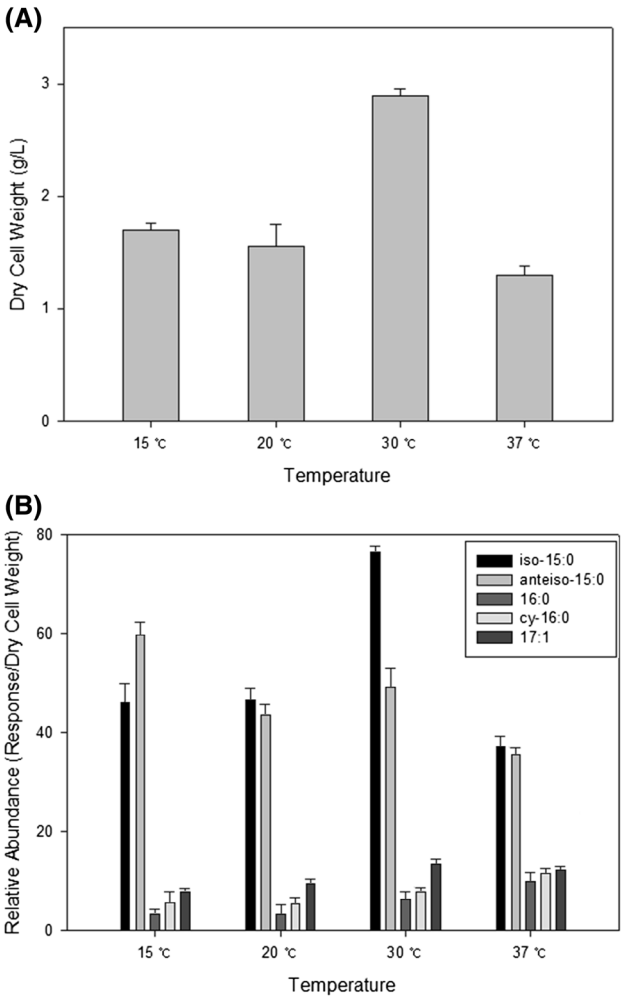

Fig. 4 Profiling of major fatty acids produced by *Bacillus pumilus* 23174 at different temperatures. **a** Dry cell weight at 15, 20, 30, 37 °C and **b** Fatty acids profiles at 15, 20, 30, 37 °C

response to orlistat addition were represented in Fig. 5. The relative quantities of the other fatty acids exhibited a little change (Fig. 5a), when the dramatic decrease of iso-15:0 and anteiso/iso-form ratio. The controlled synthesis of the anteiso/iso ratio at different temperatures in *B. pumilus* is one of the adaptive mechanisms at low temperatures. The results of the lipid inhibitor experiment clearly showed that the FAS inhibition seems to be a major function in cold adaptation, and critically affects the amount of iso-15:0. The anteiso/iso ratio of fatty acids in *B. pumilus* was balanced by controlling the amount of iso-15:0.

In *B. subtilis*, the fatty acid profile was dominated to a large extent (>80 %) by the odd-numbered iso- and anteiso-branched chain fatty acids with the major species of iso-, anteiso-15:0 and 17:0 [14, 15]. *B. pumilus* mainly contained 15:0 and detailed control was exerted through changes in the anteiso- and iso- ratio. Previously, cerulenin has been applied to control the fatty acids synthesis which

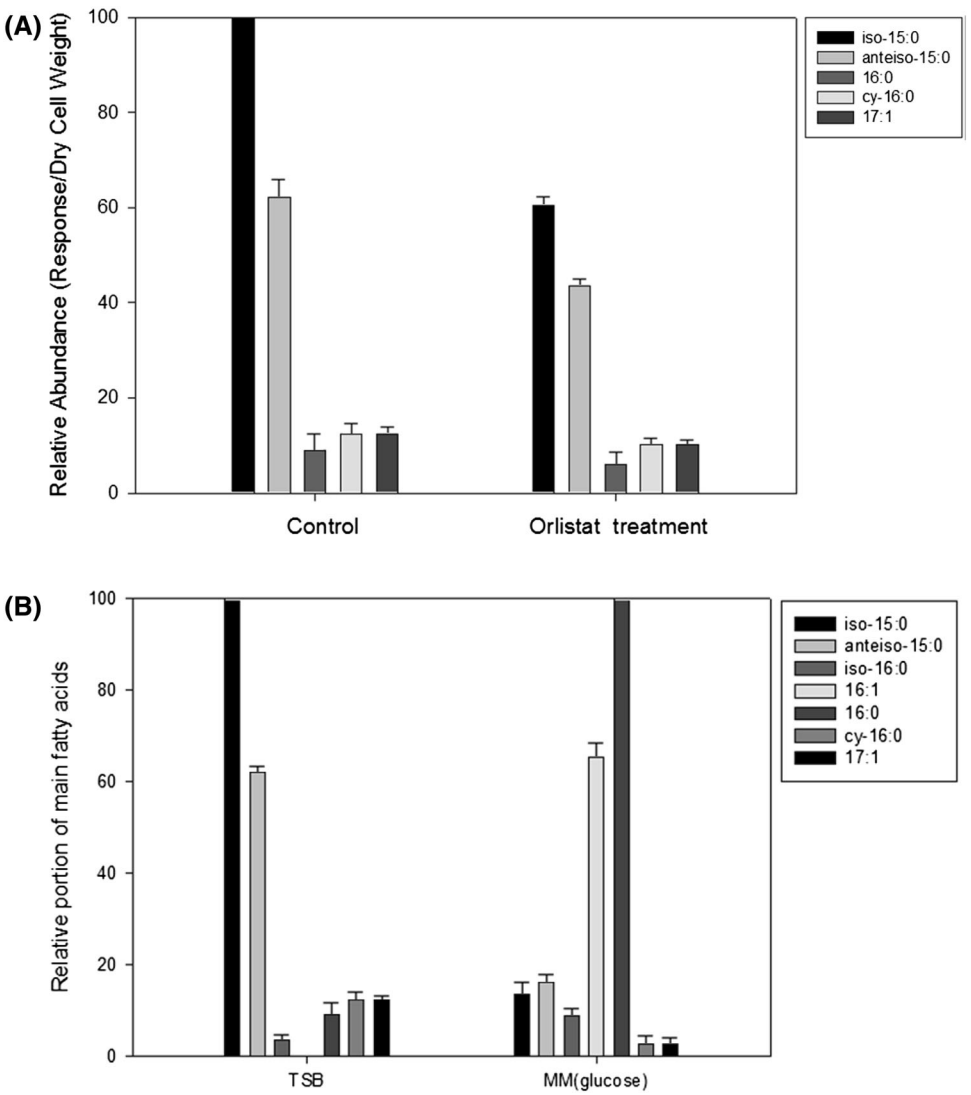

Fig. 5 Change of fatty acid profile in *Bacillus pumilus* 23174 with lipid inhibitor treatment (Orlistat) (**a**) and nutrient depletion (**b**)

inhibits the ß-ketoacyl-acyl carrier protein synthase (FabF) and it may induce the fatty acid chain shortening which usually occurs only at low temperatures. The less-expressed or less-active FabF in cold conditions or its inhibition by cerulenin may slow the fatty acid chain elongation reaction, thereby resulting in shorter fatty acids in the membrane phospholipids [16, 17]. Similar kind of reflection has been observed from our study in which, orlistat inhibits the iso-C15 form fatty acids at cold temperature and further it may affects the adaptation of *B. pumilus* at cold climate.

Change of FAME profile by adding amino acids as precursors for branched chain fatty acids in Bacillus pumilus

Amino acids precursors (Leu, Ile, and Val) were added to the original media (TSB) to promote the synthesis of iso-15:0, anteiso-15:0 and iso-16:0, respectively, to investigate the importance of exogenous nutrient sources in the production of branched chain fatty acids [12, 18, 19]. According to the previous reports, when amino acid precursors were added as free acids into a nutrient-poor medium, they are utilized for the synthesis of protein rather than synthesis of branched-form fatty acids [11, 20, 21]. At the same time, when amino acids were abundant in the medium and certain amino acid is present in extremely large quantities, the synthesis of branched chain fatty acids will be spontaneously increased [5, 22]. In our study, the fatty acid profile from the minimal media was peculiar with 16:0 fatty acids which are the major component while comparing with other fatty acids and it was entirely contrast with the fatty acid quantities of TSB media (Fig. 5b). The obtained results showed that the synthesis of iso-15:0 and anteiso-15:0 was much higher when the nutrients were abundant in the production medium. On the other hand,

when nutrients were depleted, there was a tendency to increase the amounts of 16:1 and cy-16:0 rather than iso-15:0 and anteiso-15:0.

The addition of precursors like leucine, isoleucine, and valine into the TSB media results the different levels of iso- and anteiso-15:0 and the total synthesis of 15:0 was quite lesser than the normal state (Fig. 6). This interesting fact was further confirmed by additional experiments where leucine, isoleucine, and valine were added individually. When the large amount of leucine was added, the anteiso/iso ratio was 0.86, and iso-15:0 tended to increase since that amino acid as the precursor (Table 2). The experiments with isoleucine showed that it may affect the relative amount of anteiso-15:0 and the anteiso/iso ratio were increased up to 1.53. Similarly, the precursor of iso-16:0 (valine) has showed an anteiso/iso ratio about 1.28. When the large amount of all three amino acids was added into the medium which results the anteiso/iso ratio was 0.96 and it seems to indicate that each precursor of the branched chain fatty acids has their own combinatorial effects.

The branched chain keto acid dehydrogenase (Bkd) complex converts the branched chain keto acids into branched chain acy-CoAs, which plays an important role in the final synthesis of branched chain fatty acids [23–25]. Bkd in *B. subtilis* showed a relatively higher activity in response to isoleucine (100 %) derived substrates than to valine (75 %) and leucine (31 %) derived substrates. In contrast, there was a higher preference for leucine was observed in *B. pumilus*, which is distinctive from the previous research studies [15, 26]. Therefore, this polar bacterium can sensitively produce iso-15:0 and it is also a dominating factor in the control of iso/anteiso balance. The cold shock experiments were employed to check whether the composition of the provided amino acids affects the

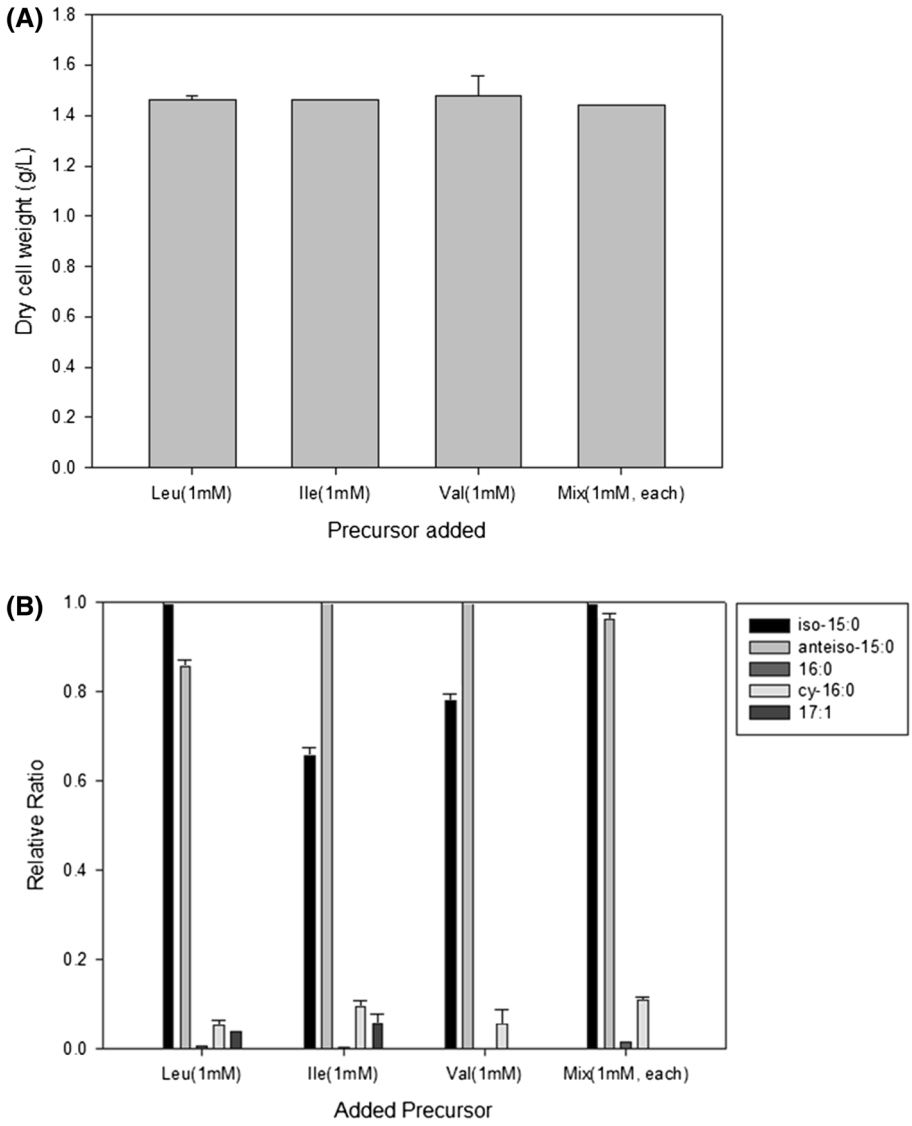

Fig. 6 Dry cell weight and fatty acid profile of *Bacillus pumilus* 23174 when the amino acids (leucine, isoleucine, and valine) were supplied as the precursors for branched chain fatty acids

Table 2 The ratio of anteiso- and iso- form fatty acids produced by *Bacillus pumilus* PAMC 23174 in each medium

Media	Growth temperature (°C)	Percentage of total fatty acid (%)					Anteiso/Iso ratio
		Iso-15:0	Anteiso-15:0	C16:0	Cy-C16:0	C17:1	
TSB (Leu)	30	51.0 ± 1.23	43.7 ± 2.04	0.4 ± 0.02	2.8 ± 0.11	2.1 ± 0.03	0.86
TSB (Ile)	30	36.3 ± 1.10	55.3 ± 1.95	0.2 ± 0.01	5.2 ± 0.21	3.1 ± 0.07	1.53
TSB (Val)	30	42.5 ± 1.41	54.4 ± 2.69	–	3.1 ± 0.16	–	1.28
TSB (Leu, Ile, Val)	30	47.9 ± 2.03	46.1 ± 1.51	0.8 ± 0.10	5.2 ± 0.09	–	0.96
TSB (Leu), cold shock	30	53.4 ± 2.11	46.1 ± 1.90	0.4 ± 0.05	0.1 ± 0.01	0.1 ± 0.01	0.86
TSB (Ile), cold shock	30	31.7 ± 1.45	67.6 ± 3.56	0.5 ± 0.04	0.2 ± 0.01	–	2.13
TSB (Val), cold shock	30	38.3 ± 0.77	61.2 ± 1.57	0.3 ± 0.05	0.1 ± 0.01	0.2 ± 0.01	1.60
TSB (Leu, Ile, Val), cold shock	30	36.6 ± 0.98	61.9 ± 2.32	0.6 ± 0.03	0.7 ± 0.02	0.2 ± 0.01	1.69

bacterial responses according to cold shock. When the cells were treated by cold shock, the anteiso/iso ratio was increased in all samples except leucine-added sample and also the medium with only leucine exhibits anteiso/iso ratio about 0.86 after the cold shock treatment. This might be due to leucine being as a precursor for iso-form fatty acid synthesis with 15 and 17 carbons. In previous reports, anteiso-form fatty acids were increased in order to withstand at low temperatures, but the change in the ratio was relatively trivial when compared to other bacterial strains. The overall nutritional effect especially leucine addition seemed to be superior to the effect of temperature by cold shock.

Conclusions

In this study, we investigated that, how bacteria can live and change their metabolic activities in polar environment at different temperatures. The polar bacterium *B. pumilis* exhibits a unique and simple lipid pattern when compared to other polar and non-polar strains and its iso-15:0 syntheses was sensitively changed in response to varying environmental conditions which further affects the balance of anteiso- and iso-form fatty acids. The synthesis of simple pattern of total fatty acids in *B. pumilus* is unclear and this strain could be useful to study about the changes of fatty acid synthesis in polar bacteria due to the different environmental conditions and nutritional fluctuations. Further, this study also provides evidence for the adaptation of bacteria in an extreme condition and their metabolic rearrangements.

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