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Effect of Age on the Fatty Acid Composition of the *Bacillus subtilis* PO270 Isolated from Wheat Rhizosphere

ROBERT ŻARNOWSKI[1,*], RICHARD J. ELLIS[2], TERESA LEWICKA[1] and STANISŁAW J. PIETR[1]

[1] Department of Agricultural Microbiology, Agricultural University, Wrocław, Poland
[2] NERC Centre for Population Biology, Imperial College at Silwood Park, Ascot, Berkshire, UK

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Abstract

The changes of the composition of growing medium and the fatty acid composition of *Bacillus subtilis* PO270, a bacterium isolated from the wheat rhizosphere, was evaluated during different phases of growth. During growth alkalinity reaction of medium was observed and in late stationary phase of growth the release of proteins and phenolic acids from cells was observed. Twenty six fatty acids were detected. The most prominent fatty acids found in bacterial cells were 15:0 *a*, 15:0 *i*, 17:0 *a* and 17:0 *i*. Depending of a phase of bacterial growth, their contents varied from 86.5 to 88.9% of total fatty acids. The remaining fatty acids identified, including regular saturated and monounsaturated as well as iso- and anteiso-branched, 2- and 3-hydroxylated, cyclopropane and odd-numbered derivatives, were present in minor amounts. We have demonstrated that the fatty acid composition of this bacterium changes greatly in different growth phases. These structural changes represent re-arrangement of membranes, which keeps the bacterial cell fit during growth and counteracts the effects of the changing environment.

Key words: *Bacillus subtilis*, fatty acids

Introduction

The *Bacillus* is an extremely diverse genus of spore-forming gram-positive bacteria exhibiting an array of physiological abilities. Bacilli can be thermophilic, psychrophilic, acidophilic, alkaliphilic, halotolerant, or halophilic and are capable of growing at different pH values, temperatures and salt concentrations. These features allow them to survive in a wide range of habitats including many extreme niches such as desert sands, hot springs or soils in Polar Regions. Bacilli are also able to colonize a variety of environments ranging from soil to insects and human organisms. Although the many well-known species of bacilli are pathogens, several *Bacillus* species are saprophytes living off of decaying organic matter. Several species of *Bacillus* genera inhabit the rhizosphere, the interface between plant roots and the surrounding soil (*e.g.* Lievens *et al.*, 1989; Foldes *et al.*, 2000; Macrae *et al*., 2001). These bacteria are able to secrete antibiotic substances that destroy neighbouring microbes (*e.g.* Lievens *et al.*, 1989; Reddy and Rahe, 1989), so that their contents may be released and digested allowing the antibiotic-producing bacilli to survive until conditions are more favourable. *B. subtilis* is one of the most studied gram-positive bacteria. Lipid metabolism and its regulation in this bacterium has been attracting an attention of researchers for a long time. There are many published reports concerning studies of various fatty acids in both vegetative cells and spores of *B. subtilis* (*e.g.* Bischop *et al.*, 1967; Kaneda, 1972; Clejan *et al.*, 1986) as well as describing effects on *B. subtilis* lipid biochemistry caused by thermal (*e.g.* van de Vossenberg *et al.*, 1999; Weber *et al.*, 2001; Altabe *et al.*, 2003), pH (*e.g.* Yumoto *et al.*, 2000), salt (*e.g.* Sakamoto and Murata, 2002) or osmotic stresses (*e.g.* Svobodova *et al.*, 1988; Lopez *et al.*, 2000). However, to our knowledge, there is no

* Present address of the author for correspondence. Dr Robert Żarnowski, Department of Medical Microbiology & Immunology, University of Wisconsin-Madison, 416 Medical Sciences Center, 1300 University Avenue Madison, WI 53706, USA Fax: ++1-608-265 6717. E-mail: rzarnowski@wisc.edu

information available concerning fatty acid composition during vegetative growth of *B. subtilis* strains of soil origin. The goal of this study was to characterize the effect of age on the total fatty acid composition of *B. subtilis* isolated from the wheat rhizosphere.

Experimental

Materials and Methods

Microbiological material. The *B. subtilis* PO270 strain was previously isolated from the rhizosphere of winter wheat cultivar Kobra (Żarnowski, 2002) and is deposited at the Microbial Culture Collection of Agricultural Microbiology Department, Agricultural University of Wrocław, Poland. Trypticase Soy Broth (TSB, Difco, Detroit, USA), $^1/_{10}$ strength, pH 7.0 was used to cultivate bacteria. Cultures were grown in 100 mL Erlenmeyer flasks containing 30 mL of medium and aerated by rotary shaking (150 rpm) at 28 ± 1°C in the dark. Cells were centrifugated (10000 × g, 5 min) after 24, 48 and 72 hours of growth at 4°C. These time points correspond to logarithmic, early stationary and late stationary growth phases, respectively. Bacterial pellets were washed with 0.1 M $MgSO_4$, re-centrifuged and freeze-dried. The experiment was done in five replications.

Analysis of cultures during growth. Amounts of soluble proteins, reducing sugars and phenolic acids as well as pH in medium before inoculation with tested strain as well as in supernatants after 24, 48 and 72 hours of growth were determined. The yield of cellular proteins in each time point was also evaluated. Content of proteins in supernatants and bacterial pellets were determined according to Bradford (1976) using bovine albumine as a standard. The amount of reducing sugars in supernatants was determined using the Somogyi-Nelson method (Nelson, 1944) using glucose as a standard. The content of phenolic acids was determined according to modified method of Krygier *et al.* (1972). For this purpose the 25 mL of tested supernatants were acidified with 0.1 N H_2SO_4 to pH 2.0 and consequently were extracted five times with equal volume of mixture of ethyl ether: ethyl acetate (1:1 v/v). Organic phases were collected and evaporated to the dryness under nitrogen and residues were dissolved in 5 mL of ethyl acetate. The amount of phenolic acids was determined spectrophotometrically at 320 nm using ferrulic acid as a standard (Sigma-Aldrich, USA).

Cellular fatty acid analysis. Approximately 50-mg freeze-dried, cells were placed in a glass tube with teflon seal. Fatty acid methyl ester preparation and analysis were carried out as described elsewhere (Thompson *et al.*, 1993). Briefly, fatty acids were saponified in alkaline methanol and subsequently methylated with hydrochloric acid in methanol. Fatty acid methyl esters were extracted with a hexane : methyl-*tert*-buthyl ether solvent mixture. Fatty acids were identified by gas chromatography using an Agilent 6890 instrument (Agilent Technologies, Palo Alto, CA) equipped with a 25 m × 0.2 mm × 0.33 µm phenyl methyl silicone column and a flame ionisation detector. Methyl esters of fatty acids were identified as described elsewhere (Wilson *et al.*, 2004) by comparison of retention times with a set of authentic fatty acid standards provided by the Sherlock® Microbial Identification System (Microbial ID Inc., Newark, DE). Abundance of fatty acids was calculated from relative peak areas. Fatty acids are named according to the number of carbon atoms followed by a colon and the number of double bonds in the molecules. Cis and trans isomers are indicated by the *c* or *t*, respectively. Iso- and anteiso-branched fatty acids are indicated by the *i* or *a* suffixes, respectively. The *cyc* suffix indicates the presence of a cyclopropane ring in the carbon chain. *Sif* means "Sum in feature" and indicates that a particular peak is a combination of two or more fatty acids in unknown proportions, but having indistinguishable retention times.

Statistics. The data obtained were processed using Statistica for Windows version 5.1 (StatSoft Ltd., London, UK). Results of pH and chemical analyses were subjected to one-way analysis of variance, and the means were separated with an FLSD ($P = 0.05$). For evaluation results of cellular fatty acid analysis two general statistical approaches were used in this study: cluster analysis and principal component analysis. Cluster analysis was used to classify the objects examined into groups (clusters), and dendrograms were constructed using most probably the most popular Ward's amalgamation algorithm. This algorithm is based on measurements of the distance between clusters utilizing analysis of variance approach (Ward, 1963). The distance between clustered objects was measured using simple Euclidean distances. Principal component analysis (unrotated principal component loadings) was applied as an alternative method to cluster analysis, however offering somewhat different possibilities of data evaluation (Tranter, 2000). This analysis was applied in order to check which of considered variables were significant and decisive for this study. In both cases the proper matrices were constructed on the basis of total fatty acid profiles specific for each of bacterial culture stages examined.

Results and Discussion

Cells of *B. subtilis* PO270 were grown through late stationary phase and changes of pH, amount of proteins, reducing sugars and phenolic acids in supernatants as well as the yield of cellular proteins and their fatty acid compositions were analyzed. The results of chemical studies of supernatants and yield of cellular proteins are shown in Table I. During logarithmic growth phase alkaline reaction of medium was observed, which was furthermore increased during early stationary phase. Available nutrients in the medium, sugars and proteins, were practically utilized completely during first 24 hrs of growth of the *B. subtilis* PO270 strain. The yield of cells, measured by the amount of proteins in cell pellets, reach 88.5% of maximum during first 24 hrs and correlated with utilization of nutrients from medium. The yield of proteins found in the cell pellet during late stationary phase significantly decreased in comparison with earlier stage (48 hrs) and this correlated with the appearance of soluble proteins in supernatants. Gohar *et al.*

Table I
Chemical characteristics of supernatants and the yield of cellular proteins of *Bacillus subtilis* PO270

Growth phase	Supernatants				Cells
	pH	Reducing sugars [mg ml^{-1}]	Proteins [mg ml^{-1}]	Phenolic acids [mg ml^{-1}]	Proteins [mg ml^{-1}]
Control medium	7.32 c*	5.9 a	15.7 a	18.4 c	0.0 b
Logarithmic	8.17 b	0.3 b	1.1 c	21.7 b	33.2 a
Early stationary	8.68 a	0.5 b	1.6 c	36.2 a	37.5 a
Late stationary	8.63 a	0.5 b	5.4 b	36.4 a	33.8 a

* values in the columns followed by the same letters are not significantly different according to Student t-test at P = 0.05

(2002) also indicated that, quantitatively, most of the proteins were secreted by *B. cereus* at the stationary phase. Secretion of proteins is related to the formation of bacterial endospores. Endospores are encased in a complex protein coat that confers protection against noxious chemicals and influences the germination response (Serrano *et al.*, 1999). Moreover, the significant increase of free phenolic acids in the supernatant was observed in early and late stationary growth phases. The amount of phenolic acids in supernatant increased from 21.7 after 24 hours to 36.2 μg per mL after next 24 hours. A similar amount was found after 72 hours of growth. This observation could indicate transformation of vegetative cells into spore forms and the corresponding melanization (Claus, 2003).

The fatty acid compositions of harvested cells are shown in Table II and Figure 1. The major fatty acids in the *B. subtilis* PO270 strain were 15:0 *a*, 15:0 *i*, 17:0 *a* and 17:0 *i* and their content varied from 86.5 to 88.9% of the total fatty acid pool. The PO270 strain showed many differences in the fatty acid composition depending on the growth phase. The main changes were observed for branched fatty acids. The amount of iso-branched fatty acids increased from 46.2% after 24 hours to 49.7% after next 24 hours and then fell to 42.3% after 72 hours of growth. A similar phenomenon was observed for regular straight saturated and unsaturated fatty acids, however concentrations of both of these fatty acid classes were considerably lower. Saturated fatty acids increased from 3.6% at logarithmic phase to 4.1% during early stationary phase and then their level fell to 3.4% in the late stationary growth phase. In the case of unsaturated fatty acids, the content was slightly increased after 48 hours and finally reached 2.6%. The content of anteiso-branched

Table II
Fatty acid composition of *Bacillus subtilis* PO270[1]

Fatty acid	Growth phase			Fatty acid	Growth phase		
	Logarithmic	Early stationary	Late stationary		Logarithmic	Early stationary	Late stationary
9:0	*nd*	*t*	*nd*	15:0 2-OH	0.1	0.1	0.1
12:0	0.1	0.1	0.4	17:1 *i* ω10*c*	0.5	0.6	0.3
13:0 *i*	0.2	0.3	0.1	*Sif 4*	0.2	0.3	0.2
13:0 *a*	0.1	0.1	0.1	17:0 *i*	9.2	9.3	8.3
14:0 *i*	1.1	1.4	0.9	17:0 *a*	9.1	7.4	9.4
14:0	0.3	0.4	0.5	17:0 *cyc*	0.1	*nd*	0.10
15:0 *i*	31.4	34.4	29.4	*Sif 5*	0.2	0.2	0.3
15:0 *a*	38.3	35.5	41.8	18:0 *i*	*nd*	*t*	*nd*
15:0	0.1	0.1	*nd*	18:1 ω9*c*	1.6	1.5	1.5
16:1 ω7*c* [2]	0.1	0.2	0.1	18:1 ω7*c*	0.6	0.6	0.6
16:0 *i*	3.3	3.0	3.1	18:0	0.3	0.3	0.3
16:1 ω11*c*	0.3	0.5	0.1	17:0 *i* 3-OH	0.1	0.1	*nd*
Sif 3	*nd*	0.2	0.2	17:0 2-OH	0.1	0.1	*nd*
16:0	2.8	3.2	2.2	19:0	*nd*	0.1	*nd*
15:0 *i* 3-OH	0.1	0.1	0.1				

[1] values represent relative percentage composition; [2] as alcohol; *nd* – not detected; *t* – trace (below 0.05 %)

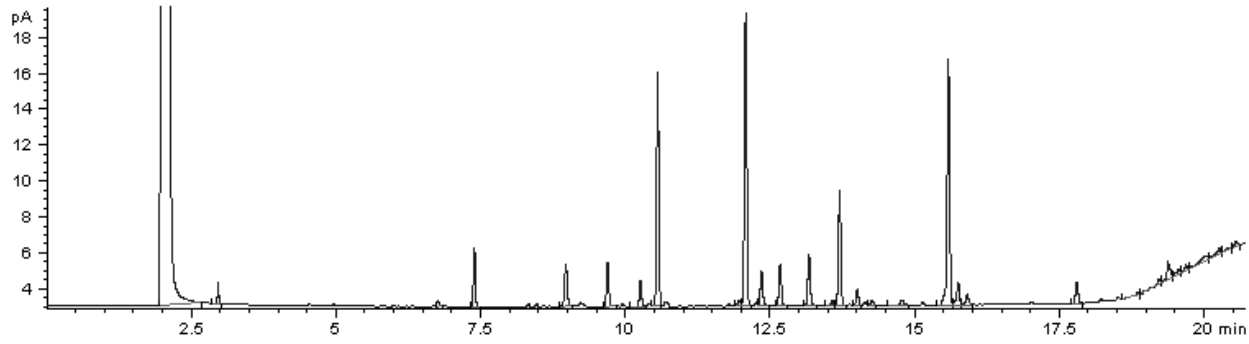

Fig. 1. An example of gas chromatogram obtained after separation of fatty acid methyl esters from *Bacillus subtilis*

fatty acids decreased from 47.5% to 43.0% and after that reached a level of 51.3% of the total fatty acid pool. Interestingly, a level of all of those fatty acids was invariable during the *B. subtilis* growth. The variability observed for hydroxy, cyclopropane and odd-numbered fatty acid derivatives was not of importance, because those fatty acids were found in insignificant quantities only. These findings are in a good agreement with fatty acid patterns reported previously for this bacterial species (Holmgren, 1978; Suutari and Laakso, 1992).

The changes observed in the fatty acid composition of *B. subtilis* might be to ensure the integrity of the biological membranes and to prevent unwanted mismatch of lipids during the growth. The ratio of anteiso- to iso-branched fatty acids was initially increased by *ca.* 1.2-fold and then considerably reduced reaching a value of 0.86. The level of main saturated fatty acids (including all straight and branched) was constant during the monitored growth phases of the *B. subtilis* PO270 isolate and amounted to about 96.5%. Similarly, a ratio of membrane unsaturated to saturated fatty acids remained at the same level (*ca.* 0.8%) and was only slightly reduced with age. Ratios of hydroxy fatty acids to either saturated or unsaturated fatty acids were also decreased, however these changes could not be considered as significant, because of a very low content of hydroxy fatty acids in *B. subtilis* cells. The level of iso- as well as anteiso-branched to hydroxy fatty acids was considerably increased with bacterial culture age. This suggests that membrane fluidity changes when the cells grown at higher pH and during spore formations. Similar increase of anteiso-/iso- branched fatty acid ratio described Yumoto *et al.* (2000) in an alkaline environment.

Statistical analyses were performed to investigate the effect of age on the fatty acid composition in *B. subtilis* PO270. This approach was also used to demonstrate which of fatty acids varied significantly with age. Cluster analysis proved the presence of significant differences amongst the analyzed fatty acid profiles. The maximum value of Euclidean distance calculated was 7.187163 (100%), whereas the value was 4.267785 (59.4%) for a cluster grouping logarithmic and late stationary growth phases. The early stationary growth phase was found to be considerably different from the two other stages examined, mainly due to changes in a ratio of iso-/anteiso-branched fatty acids. Unfortunately, principal component analysis could not be used for such analysis, because it yielded only one principal component explaining *ca.* 97% of the total variance in the data (not shown). This method was found, however, to be appropriate to demonstrate which of considered fatty acids were significant and decisive for this study. In this case, principal component analysis yielded two principal components (PC1 and PC2) that explained above 70.0 and 24.8% of total variance in the data processed. The first principal component was correlated well with almost all identified fatty acids with the exception of 14:0, 16:0 *i*, *Sif* 3, 18:1 ω9*c* and 18:0, which correlated with the second principal component (Fig. 2). Values calculated for those principal components varied from –0.9757 to 0.9758 and from –0.9748 to 0.8934 (for PC1 and PC2, respectively), which indicates the presence of both positive and negative correlations. All analyzed fatty acids excluding the mentioned 14:0, 16:0 *i*, *Sif* 3, 18:1 ω9*c* and 18:0 were included into the same cluster and form the same group. A similar observation has been gathered with respect to those five fatty acids correlated with PC2. Because principal component analysis is invariant to the mirroring through the origin (Tranter, 2000), there is no need to evaluate all of these variables to achieve the same level of characterization of the objects examined. Therefore it would be enough to afford only one or few fatty acids correlating with particular principal components.

Observed increase of pH and accumulation of phenolic acids with simultaneous decrease of soluble proteins and sugars in medium significantly changed the osmotic pressure as well as redox potential. Both the osmotic pressure and the redox potential necessitate rearrangement of biological membrane structures

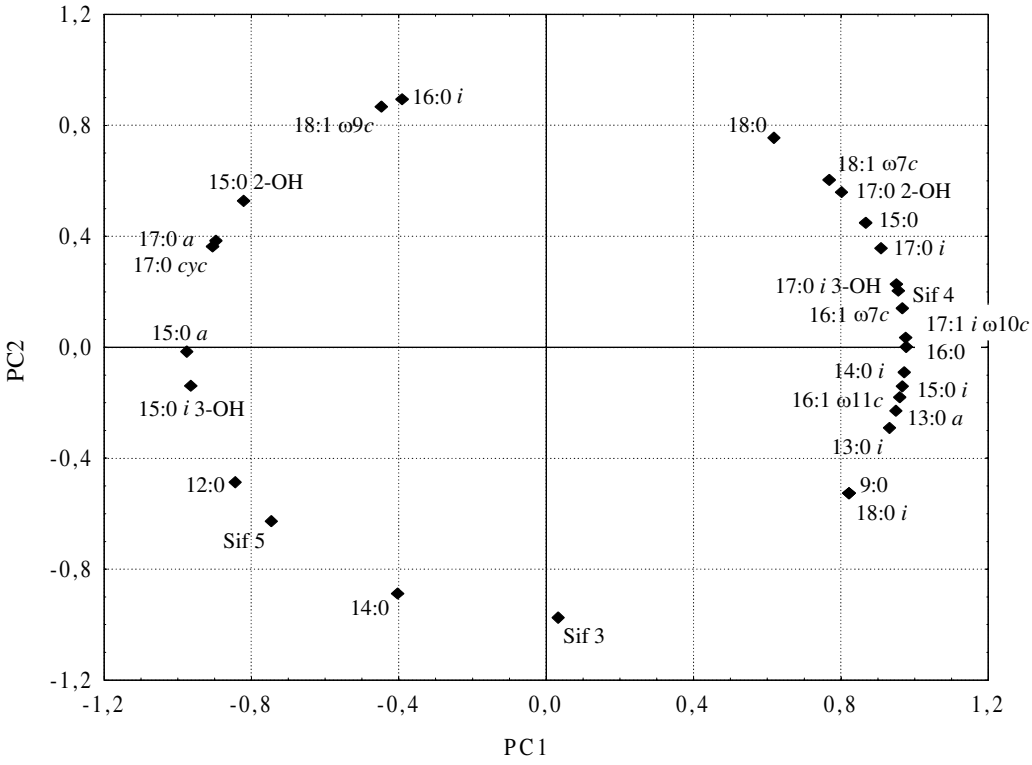

Fig. 2. Grouping of *Bacillus subtilis* PO 270 fatty acids using principal component analysis (unrotated principal component loadings) based on calculated principal components PC1 and PC2 of Ward's amalgamation algorithm by means of simple Euclidean distances.
The scale represents real (absolute) values

and variability observed might be a result of such rearrangement of membranes. This would keep the growing bacterial cell physiologically fit during growth and counteract the effect of the changing environment. Each lipid molecule possesses its own unique shape, which depends on a type of substituted fatty acid chains, and therefore different lipid classes can form desirable biological membrane structures and maintain their properties (Cronan and Felmann, 1975; Russell and Fukunaga, 1990; Kaneda, 1991).

However, in order to correlate our herein presented findings on age-dependent changes in cellular fatty acid patterns, further studies of gel-to-liquid phase transition of membrane lipids in this strain would be of great interest.

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