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Development of Membrane Lipids in the Surfactin Producer *Bacillus subtilis*

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ABSTRACT. Processes occurring in the cytoplasmic membrane of the surfactin producer *Bacillus subtilis* were examined during a 3-d cultivation. The fatty acid composition was found to be almost stable within this interval, except for the early stationary phase when the nonbranched, mostly $C_{16:0}$ and $C_{18:0}$ (high melting fatty acids), prevailed transiently in the membrane. As for phospholipids, phosphatidylglycerol and phosphatidylethanolamine, representing 73 % of the total in the membranes of exponential cells were partly replaced by cardiolipin, which gradually rose from 5 to 28 % at the end of cultivation. In parallel, steady-state fluorescence anisotropy (r_S) measurements with 1,6-diphenyl-1,3,5-hexatriene (DPH) indicated a remarkable increase of r_S DPH during the long-term cultivation and implied a continuous rigidization of the membrane interior. By contrast, the almost constant values of r_S 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene 4-toluenesulfonate (TMA-DPH) reflected stable microviscosity of the membrane surface region. Thus, the significant increase of high melting fatty acids and cardiolipin in the cytoplasmic membrane together with the progressive rigidization of the membrane interior reflected the cell adaptation to adverse conditions.

Abbreviations

CL	cardiolipin	PG	phosphatidylglycerol
DPH	1,6-diphenyl-1,3,5-hexatriene (all *trans*)	PMSF	phenylmethanesulfonyl fluoride
FA(s)	fatty acid(s)	PS	phosphatidylserine
GC–MS	gas chromatography–mass spectrometry	$PXNH_2$	lysylphosphatidylglycerol (*see* footnote *d* to Table II)
HPLC	high performance liquid chromatography	r_S	steady-state fluorescence anisotropy
PA	phosphatidic acid	TMA-DPH	1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene 4-toluenesulfonate
PE	phosphatidylethanolamine		

Bacillus subtilis as a soil-dwelling organism encounters strong physical and nutritional fluctuations related to this hostile environment (Strauch 1993). A substantial part of adaptive processes occurs at the level of cytoplasmic membrane that functions as a selective interface between the cell and its environment. *B. subtilis* possesses efficient mechanisms to control both the FAs and polar head group composition in response to harsh growth conditions (Mansilla and de Mendoza 2005). However, the contribution of different lipids to this structure–function relationship in the bacterial membrane is not fully understood.

Upon entry into stationary phase, *B. subtilis* cells start to produce a lipopeptide antibiotic surfactin, a membrane-active agent with a wide antimicrobial activity and technical applications (Cameotra and Makkar 2004; Mulligan 2005; Rodrigues *et al.* 2006; Seydlová and Svobodová 2008). Surfactin is composed of a cyclic heptapeptide moiety with the typical sequence Glu–Leu–D-Leu–Val–Asp–D-Leu–Leu which is closed to a lactone ring by a $C_{12–16}$ 3-hydroxy FA (Kakinuma *et al.* 1969). This structure obviously explains the amphipathic nature and exceptional surface properties of surfactin (Maget-Dana and Ptak 1995). Surfactin also displays a strong membrane-destabilizing action at concentrations even below its critical micellar concentration and induces the formation of ion channels in lipid bilayers (Heerklotz and Seelig 2007). In other words, it could be hypothesized that the *B. subtilis* cytoplasmic membrane may encounter a combined stress of the stationary-phase conditions and its own product at the same time.

To understand deeper the physiological role of the membrane lipids in the surfactin producer *B. subtilis*, the biochemical and biophysical parameters of the cytoplasmic membrane were monitored here. The development of both the membrane polar head groups and FAs was analyzed and the kinetics of surfactin synthesis was assayed during a 3-d cultivation. The microviscosity of the cytoplasmic membranes determined by steady-state fluorescence anisotropy of DPH and its polar derivative TMA-DPH as fluorescence probes complemented the biochemical data.

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MATERIALS AND METHODS

Growth of bacteria. The strain *B. subtilis* ATCC 21332 (*American Type Cultures Collection*) was grown under optimum conditions in a nutrient broth (pH 7.0; *Oxoid*) with aeration (2 Hz, 3 d) at 30 °C. The growth was monitored turbidimetrically (absorbance A_{420}). After 3 h, bacterial cultures were harvested by rapid filtration through a Synpor no. 5 filter (*Pragochema*, Czechia). After 7, 12, 24 and 72 h the cells were harvested by centrifugation (3000 ***g***, 20 min, 4 °C) and used for isolation of cytoplasmic membrane fractions or lipid extraction.

Surfactin concentration was determined according to Wei and Chu (1998) with slight modifications. Cells were pelleted from the culture samples and the supernatants were analyzed by HPLC (*Agilent* 1100) equipped with a WRP-18 column (7 μm, *Watrex*). The mobile phase consisted of 3.8 mmol/L trifluoroacetic acid–water (15 : 85). Sample size was 20 μL, the elution rate being 0.6 mL/min. Surfactin (*Sigma*) served as a standard. MS was performed on *Bruker* Esquire 3000 and surfactin homologs (C_{13}, C_{14}, C_{15}) were detected at *m*/*z* 1008, 1022 and 1036, respectively. The data were analyzed by *Bruker* Daltonics Data Analyses 3.0 software.

Lipid extraction and analysis. The membrane phospholipids were isolated using the method of Radin (1981) with modifications. The cells were extracted in a hexane–2-propanol (*Lach-Ner*, Czechia) mixture (3 : 2) and stored overnight at –20 °C. The extraction mixture was centrifuged (3 000 ***g***, 5 min, 0 °C). After evaporation of the solvent *in vacuo* at 40 °C, the isolated phospholipids were analyzed by TLC on silica gel 60 G plates (*Merck*) in chloroform–methanol–water (65 : 25 : 4, *V*/*V*/*V*) as the mobile phase. The spots were detected with iodine vapor and identified by comparison with authentic purified standards (*Sigma*). To localize the amino groups the plates were treated with 0.2 % ninhydrin solution (1-butanol–acetic acid 19 : 1, *V*/*V*). The lipid phosphorus content was quantified according to Rouser and Yamamoto (1970).

Fatty acid analysis. To determine the FA composition, the methyl esters were prepared from isolated phospholipids by trans-esterification with sodium methoxide and HCl in methanol (Glass 1971). The methyl esters were analyzed by GC–MS using a *Varian* 3400 gas chromatographer coupled to a *Finnigan* INCOS 50 mass spectrometer equipped with a DB5 column and *ProLab Resources* software. A mixture of bacterial FA methyl esters (*Supelco*) was used as standard.

Isolation of membranes. Cytoplasmic membranes were isolated using enzymic lysis (Bisschop and Konings 1976). Cells (4 g wet mass per L) resuspended in phosphate buffer (50 mmol/L, pH 8.0; 30 °C) with PMSF (1 mmol/L; *Serva*) were incubated with lysozyme, DNAase, RNAase (all *Sigma*) at a concentration of 300, 10 and 10 μg/mL, respectively, and $MgSO_4$ (10 mmol/L) for 1 h at 30 °C. Then EDTA (15 mmol/L) and after 1 min $MgSO_4$ (10 mmol/L) were added. The cell lysate was centrifuged (3000 ***g***, 10 min, 4 °C) to remove the crude cell debris. The cell-free supernatant was centrifuged (12 000 ***g***, 1 h, 4 °C). The membrane sediment (10 mg of membrane proteins per mL) in a phosphate buffer (100 mmol/L, pH 6.6) was stored at –70 °C. The protein concentration was determined by the bicinchoninic acid (*Pierce*) method (Smith *et al.* 1985).

Measurement of r_s. DPH and TMA-DPH were purchased from *Molecular Probes* (USA). Measurement of r_s was performed with a Fluoromax 3 spectrofluorimeter (*Jobin Yvon*, Japan). Samples of cytoplasmic membranes (75 μg of membrane proteins per mL) were resuspended in phosphate buffer (25 mmol/L, pH 7.0) with the probe concentration of 1 μmol/L; then they were incubated for 30 min at 37 °C. Excitation and emission wavelengths were measured at 360 and 450 nm, respectively. Temperature of r_s DPH and r_s TMA-DPH measurements was identical with the temperature of cultivation (30 °C). The intrinsic fluorescence intensity of unlabeled membranes did not exceed 1 % of the experimental values; the r_s was calculated according to Lakowicz (1983).

RESULTS

Growth of B. subtilis *and kinetics of surfactin production.* The growth rate was characterized in nutrient broth at 30 °C by a doubling time of 38 min and the culture reached the stationary phase at A_{420} of 1.5 (Fig. 1). Surfactin production rose during the stationary phase (Table I, Fig. 1) reaching the final level of 120 mg/L; three surfactin homologs were found with the C_{13}, C_{14}, and C_{15} FA chain. The relative proportions of these homologs remained stable with C_{15}-surfactin as the most abundant one within the whole period of cultivation.

Fatty acid composition. During the exponential phase at 3 h the branched-chain FAs were detected as the dominant component that accounted for 77 % of the total membrane lipids. Within the wide spectrum of FAs identified in the membrane *anteiso*-$C_{15:0}$ FA prevailed, constituting 39 % of the total (Table II *top*).

Table I. Kinetics of surfactin synthesis (concentration, mg/L[a]) during 3-d cultivation (3, 7, 12, 24, and 72 h) of *B. subtilis* ATCC 21332

Surfactin homologs[b]	3	7	12	24	72
C_{13}	<0.5	9.0 ± 1.1	12.0 ± 0	14.0 ± 0.7	20.0 ± 1.4
C_{14}	<0.5	1.5 ± 0.7	6.5 ± 0.4	7.5 ± 1.8	10.0 ± 1.4
C_{15}	<0.5	34.0 ± 1.1	45.0 ± 1.4	58.0 ± 0.7	90.0 ± 0.7
Total	<0.5	44.5 ± 0.7	63.5 ± 1.8	79.5 ± 1.8	120.0 ± 3.5

[a]Average ± SD. [b]With fatty acid chain length of 13, 14, and 15 C atoms, respectively.

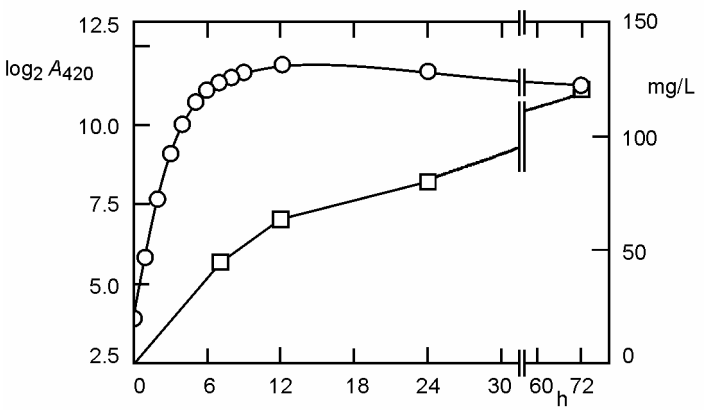

Fig. 1. Growth curve of *B. subtilis* ATCC 21332 [*circles*; $\log_2(A_{420} \times 1000)$] and the production of surfactin (*squares*; concentration, mg/L).

Table II. Fatty acid (FA) distribution (relat. %[a] ± SD) and phospholipid (PL) composition (relat. %[b] ± SD) in *B. subtilis* ATCC 21332 during 3-d cultivation (3, 7, 12, 24, and 72 h) at 30 °C

Fatty acid	*FA distribution*					
	mp[c], °C	3	7	12	24	72
14:0	54	2.0 ± 0.1	4.1 ± 0.4	1.4 ± 0.4	1.1 ± 0.2	1.5 ± 0.0
i-14:0	53	1.5 ± 0.1	0.9 ± 0.1	1.2 ± 0.2	1.4 ± 0.0	1.1 ± 0.1
15:0	53	0.9 ± 0.1	1.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	1.0 ± 0.2
i-15:0	52	11.3 ± 0.9	9.5 ± 1.3	20.6 ± 1.6	21.1 ± 1.2	23.0 ± 0.6
a-15:0	23	38.8 ± 0.7	20.0 ± 1.6	34.0 ± 2.3	36.8 ± 0.1	34.7 ± 1.3
16:0	63	9.7 ± 0.2	22.9 ± 1.6	10.8 ± 2.8	5.6 ± 0.5	6.9 ± 1.3
i-16:0	62	5.2 ± 0.5	2.2 ± 0.2	3.5 ± 0.3	4.6 ± 0.2	3.7 ± 0.4
16:1	–0.5	4.1 ± 1.7	3.9 ± 0.7	2.0 ± 0.0	2.0 ± 0.6	1.4 ± 0.1
17:0	61	0.3 ± 0.0	2.4 ± 1.1	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
i-17:0	60	5.8 ± 0.6	4.6 ± 0.7	9.3 ± 0.3	11.4 ± 1.1	9.6 ± 0.5
a-17:0	37	14.2 ± 0.1	8.5 ± 0.3	12.3 ± 1.7	12.5 ± 0.7	13.9 ± 2.0
18:0	71	2.6 ± 0.8	13.2 ± 2.1	2.5 ± 0.9	1.6 ± 0.0	1.8 ± 0.3
18:1	5	3.6 ± 0.5	6.0 ± 0.7	1.5 ± 0.7	1.2 ± 0.3	1.2 ± 0.5
Phospholipid	*PL composition*					
	3		7	12	24	72
$PXNH_2$[d]	3.2 ± 1.2		6.2 ± 0.8	2.9 ± 1.1	5.6 ± 0.4	3.9 ± 0.5
PS	16.6 ± 1.4		15.7 ± 2.7	13.6 ± 1.0	13.5 ± 0.6	12.5 ± 2.0
PG	41.5 ± 3.2		35.1 ± 2.7	45.4 ± 0.4	32.2 ± 1.6	29.9 ± 1.5
PE	31.3 ± 1.4		22.7 ± 1.7	18.7 ± 1.6	20.9 ± 1.4	22.7 ± 1.0
CL	4.7 ± 0.6		14.9 ± 2.4	16.7 ± 2.1	22.4 ± 1.2	28.0 ± 0.6
PA	2.7 ± 0.3		5.4 ± 1.9	2.7 ± 0.2	5.4 ± 0.2	3.0 ± 0.7

[a]Of a given fatty acid to the total fatty acids.
[b]Of a given phospholipid to the total phospholipids of the cytoplasmic membrane.
[c]Melting point.
[d]The phospholipid class designated $PXNH_2$ in this paper is ninhydrin-positive and is probably identical with lysylphosphatidylglycerol (lysyl-PG) (Lindgren 1981).

After 7 h, a remarkable FA reconstruction was induced in the cytoplasmic membrane. When compared with the sample of lipids isolated after 3 h, the branched-to-nonbranched FA proportion reversed from 77 : 23 to 46 : 54. In particular, the sum of the high-melting rigidizing $C_{16:0}$ and $C_{18:0}$ FA rose from 12 to 36 % of the total at the expense of the low-melting fluidizing *anteiso*-$C_{15:0}$ and *anteiso*-$C_{17:0}$ series. In contrast, after 12 h the spectrum of FA turned back to the original pattern of FA identified in the exponential cells where the branched FA represented the majority. The only exception was the enhancement of high-melting *iso*-$C_{15:0}$ and *iso*-$C_{17:0}$ series that emerged after 12 h and persisted till 3 d.

Phospholipid composition. PG and PE constitute the principal lipid components of *B. subtilis* cytoplasmic membrane that are accompanied by lower quantities of PS, CL and traces of PA and $PXNH_2$ (Table II *bottom*). This pattern continued during the first 12 h. PG remained to be the main phospholipid component of the membrane during the whole growth period although its content dropped from 42 to 30 % within 3 d. Similarly, the content of PE was reduced in the membrane from 31 to 23 %. The proportion of CL developed most dynamically; at the end of cultivation it reached 6× higher values than in the exponential cells and represented 28 % of the total.

Table III. Steady-state fluorescence anisotropy (r_S) of DPH and TMA-DPH labeled *B. subtilis* ATCC 21332 cytoplasmic membrane[a]

Time, h	r_S DPH	r_S TMA-DPH
3	0.1864 ± 0.0006	0.2555 ± 0.0020
7	0.2059 ± 0.0010	0.2632 ± 0.0017
12	0.2144 ± 0.0010	0.2571 ± 0.0013
24	0.2340 ± 0.0018	0.2583 ± 0.0028
72	0.2398 ± 0.0003	0.2662 ± 0.0005

[a]Average ± SD.

Measurement of r_S *of cytoplasmic membranes.* During the entire cultivation period (Table III) the fluorescence anisotropy data of DPH-labeled membranes exhibited a persistent increase from 0.186 to 0.240, *i.e.* enhancement of 30 %. This time course of r_S DPH suggested the increasing rigidity of the cytoplasmic membrane interior. By contrast, the r_S TMA-DPH values indicated almost stable physical properties of the membrane surface during the whole cultivation with slight peaks formed at 7 h and 3 d. Nevertheless, when compared with the respective r_S DPH values of each membrane sample, the r_S TMA-DPH data document higher rigidity of the surface region of the lipid bilayer.

DISCUSSION

Cytoplasmic membrane of *B. subtilis* underwent an extensive reconstruction during the long-term cultivation. Both the FAs and polar head groups of membrane phospholipids were affected during the growth. Branched-chain FAs, representing almost 80 % of the total in the membrane of exponential cells, were transiently reduced within the early stationary phase. Under the conditions of initiated starvation and surfactin production the nonbranched $C_{16:0}$ and $C_{18:0}$ FAs with extended chains emerged in the membrane as the dominant component. These high-melting FAs brought about membrane rigidization, which was confirmed by the higher r_S DPH values. The prolonged acyl chains together with the decrease of branched-chain FAs result in higher packing and rigidity of the membrane interior (Guerzoni *et al*. 2001; Toman *et al*. 2007). Similar stress response was also observed in *B. subtilis* membrane under hyperosmotic stress (López *et al.* 2000, 2006) and *Pseudomonas putida* exposed to different solvents (Ramos *et al.* 1997).

The most significant changes in the membrane polar head groups occurred during the stationary phase when the FA response stopped. The gradual replacement of PG and PE by CL suggests the preferential synthesis of this "stress phospholipid" *via* enzymic conversion of its precursor PG (Rigomier *et al.* 1978; Petersohn *et al.* 2001). Accumulation of CL with the concomitant decrease in the PE proportion that leads to increased cell-membrane rigidity also in *Pseudomonas putida* cells treated by solvents (Ramos *et al.* 1997) can explain the continuously growing membrane rigidity in our *B. subtilis* that is exposed to nutrient limitation and surfactin production.

A similar effect of starvation to produce an increased content of CL at the cost of PG in the early stationary phase was also found for *B. stearothermophilus* (Card *et al.* 1969), *B. cereus* (Lang and Lundgren 1970), *Micrococcus luteus* (Mukamolova *et al.* 1995), *Staphylococcus aureus*, and *E. coli* (Wanner and Egli 1990).

In the eukaryotic membrane the anionic phospholipids, such as CL, stabilize the lipid bilayer (Hoch *et al.* 1992; Lewis and McElhaney 2000). Thus the remarkable accumulation of CL observed in our strain may support the essential role of the cytoplasmic membrane in the stationary cells. In addition, CL could prevent the anionic surfactin from coming close to the surface of the membrane bilayer (Maget-Dana and Ptak

1995). However, the putative relation between the surfactin production and the extensive membrane reconstruction would need to be further analyzed.

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