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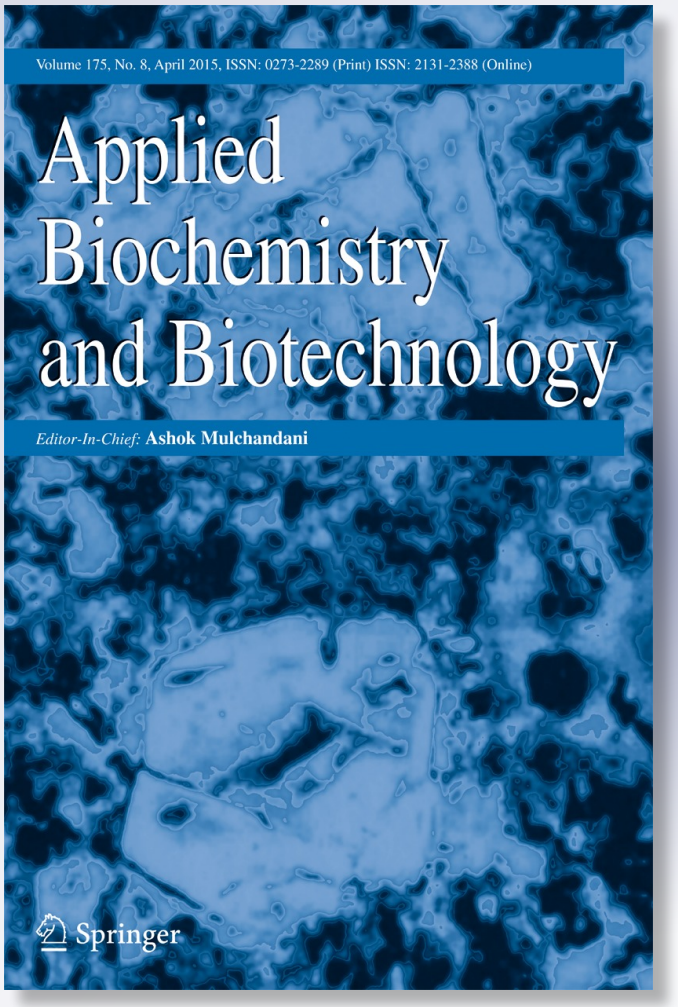

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Increased Vulnerability to Physical Stress by Inactivation of NdgR in *Streptomyces coelicolor*

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Abstract The antibiotic production and spore formation process in *Streptomyces coelicolor* need complex decision making processes by several regulatory units. These regulatory units are involved in both primary and secondary metabolism. As a result, most regulators have several functions, and those are worthwhile themes to study about different functions of a known regulator. In this study, a deletion mutant of *ndgR*, which encodes the nitrogen-dependent growth regulator, was examined by the cell viability test, TEM, and growth in *N*-acetylglucosamine/asparagine (GlcNAc/Asn) liquid medium. The results of the study show that NdgR is also involved in the structure of the cell membrane affecting survival under physical shocks. Deletion of *ndgR* leads to abnormal cell membrane resulting in the vulnerable cells to physical stress caused by shaking with beads in liquid culture condition. This empirical observation is the first meaningful explanation to why *ndgR* mutant could not grow well in a

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liquid minimal medium due to the defect of *N*-acetylglucosamine (GlcNAc) utilization and phospholipid synthesis.

Keywords *Streptomyces coelicolor* · *ndgR* · Physical stress · *N*-acetylglucosamine · Cell membrane

Introduction

Streptomyces coelicolor is soil-dwelling bacteria that evolved to survive under poor nutrient conditions. It produces spores and several secondary metabolites such as undecylprodigiosin (Red), actinorhodin (Act), methylenomycin, and calcium-dependent antibiotic [4, 10]. Naturally, it has complex systems to control these mechanisms, and many studies were performed on its critical decision making processes [3, 5, 6]. However, we have limited knowledge related to regulators, regulations on different nutrients, and irreversible antibiotic productions [2, 7, 9].

We used both the DNA affinity capture and mass spectrometric identification (DACA-MS) method [16] to find out novel regulators in a high throughput manner and screened the regulators involved in antibiotic synthetic pathways in various *Streptomyces* species. Using this approach, we identified several new regulators and started to characterize their role in processes such as NdgR, sIHF, and AbsC [17–19].

Previously, we demonstrated that the role of NdgR in *S. coelicolor* as the regulator involved in amino acid metabolisms, quorum sensing, morphological changes, antibiotic production, and NdgR was well conserved throughout the *Streptomcyes* species and many other bacteria such as *Mycobacteria* and *Corynebacteria* [12, 17]. In *S. coelicolor*, *ndgR* deletion mutant showed slow cell growth, defects in differentiation, and enhancement of the production of actinorhodin (Act) in minimal media containing certain amino acids where wild-type strain could not produce Act [17].

The wild-type strain could grow well by shaking with glass beads, which can help aeration and avoid aggregation of the cell [10] in liquid minimal medium, whereas *ndgR* deletion mutant could not grow well when it was shaken by glass beads [17]. It could grow well on solid media; however, there was no growth in liquid media by shaking with glass beads. In this study, we found that the deletion of *ndgR* results in abnormal membrane structure and vulnerability to physical stress due to *N*-acetylglucosamine (GlcNAc) utilization and phospholipid synthesis. This can be one explanation to why *ndgR* mutant could not grow well in a liquid with a minimal medium regardless of carbon and nitrogen sources, and it may help in understanding the different growth patterns of antibiotic production in liquid and solid media.

Materials and Methods

Bacterial Strains and Media

Streptomyces was cultured following the standard procedures [10]. Briefly, fresh spores of the wild-type strain (M145) and *ndgR* deletion mutant (BG11) were collected on R5 composed of 103 g of sucrose, 0.25 g of K_2SO_4, 10.12 g of $MgCl_2{\cdot}6H_2O$, 10 g of glucose, 0.1 g of Difco casamino acids, 2 mL of a trace element solution composed of 40 mg of $ZnCl_2$, 200 mg of $FeCl_3{\cdot}6H_2O$, 10 mg of $CuCl_2{\cdot}2H_2O$, 10 mg of $MnCl_2{\cdot}4H_2O$, 10 mg of $Na_2B_4O_7{\cdot}10H_2O$,

10 mg of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 5 g of yeast extract, 5.73 g of TES buffer, and 7 mL of 1 N NaOH in 1 L of distilled water. BG11 (Δ*ndgR*) was constructed as described in our previous report [17].

Culture in Liquid Medium

M145 and BG11 were cultured in liquid minimal medium under shaking condition with glass beads to improve the cell growth and antibiotic productions. To investigate the effect of the aeration and physical stress to cell growth, we observed the cell growth via three different culture conditions: (i) culture with shaking and beads, (ii) culture without shaking with beads, and (iii) culture without shaking and no beads. The cells were cultured in 25 mL minimal media containing 2.5 g (on average) of glass beads using a rotary shaker at 200 rpm at 30 °C.

Cell Viability Test

The cell membranes were stained using 0.2 μg/mL solution of FM® 5-95 (*N*-(3-trimethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium-dibromide; absorbance 560 nm/emission 734 nm) prepared in Hanks' balanced salt solution (HBSS). To analyze viability, the ratio of live/dead cells was determined by staining nucleic acids with a combination of green-fluorescent SYTO® 9 stain (480/500 nm) and red-fluorescent propidium iodide (PI; 490/635 nm) (the exCitation/emission maxima for these dyes) [19]. All the dyes were obtained from Molecular Probes, Inc (Eugene, OR, USA). Two hundred microliters of the samples was mixed with 1 μL of the 1:1 mixture of SYTO® 9 and PI and incubated for 15 min in dark. The released fluorescence was measured using a Gemini XPS fluorimeter (Molecular Devices, Sunnyvale, CA).

Transmission Electron Microscopy (TEM)

The cells grown in liquid minimal medium were harvested by centrifugation at 13,000 rpm and observed by TEM (JEM-1010, JEOL, Japan). The harvested cells were prefixed in 2 % paraformaldehyde and 2 % glutaraldehyde in 0.05 M sodium cacodylatebuffer (pH 7.2) for 2 h and then post-fixed with 1 % osmium tetroxide in sodium cacodylate buffer (pH 7.2) for 2 h. The samples were then stained in 0.5 % uranyl acetate for overnight at 4 °C and dehydrated through an alcohol series (30, 50, 70, 90, and 100 % twice each for 30 min, respectively). The samples were treated twice with 100 % propylene oxide and embedded in Spurr's resin and polymerized at 70 °C for 24 h. The embedded blocks were then sectioned using an ultramicrotome (MTX, RMC, USA), and the sections were mounted on copper grids. Eventually, the structures of samples were examined by TEM.

Results

Finding of Poor Growth of *ndgR* Mutant in Liquid Minimal Medium

When M145 and BG11 were cultured following the standard condition by shaking flasks containing the glass beads in liquid minimal medium to measure metabolites, the maximum cell mass of BG11 was observed ~2 % of wild-type M145. However, BG11 showed that the aeration and physical stress were not helpful for its growth. As shown in Fig. 1, wild type clearly shows that the best growth was by shaking with glass beads. In contrast, BG11 cultured without

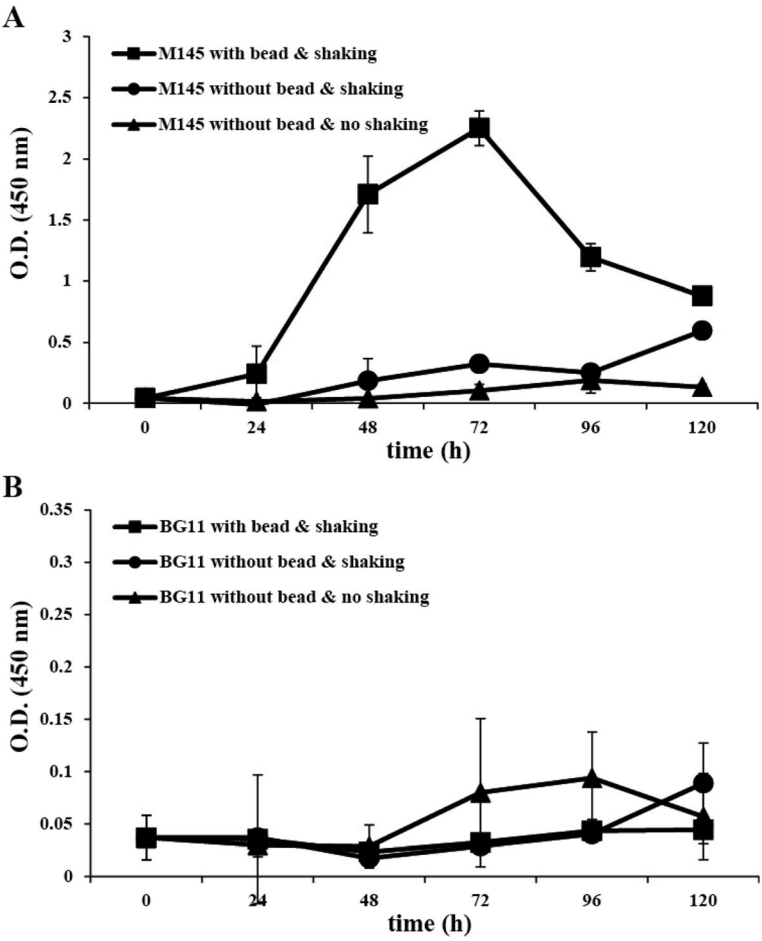

Fig. 1 Growth curves of wild types M145 (**a**) and BG11 (**b**) that were cultured in liquid MM GlcNAc/ASN. Average OD is observed at 450 nm. Cells shaken with beads, shaken without beads, and no shaking (without beads) are indicated as *filled square*, *circle*, and *triangle*, respectively. The graphs represent the average values from the biological replicate experiments (n=2). BG11 showed poor growth in liquid media, particularly shaking with beads

shaking and glass beads showed the best growth compared to other culture conditions. After 120 h, the growth decreased, resulting in death phase in minimal medium (data not shown).

Increased Vulnerability to Stress in *ndgR* Mutant

Compared to the wild-type M145, the growth of BG11 drastically decreased in liquid culture (Fig. 1). To see whether BG11 grown in liquid medium kept growing and alive, we tested the cell viability using fluorescent dye staining. To analyze the cell viability, the ratio of live/dead cells was determined by staining nucleic acids with a combination of green-fluorescent SYTO® 9 and red-fluorescent PI. The SYTO® 9 permeates and stains all types of cells, whereas PI stains only the cells with damaged cell membranes or the dead cells. The fluorescent ratio of live/dead cells is shown in Fig. 2. As expected, the physical stress such

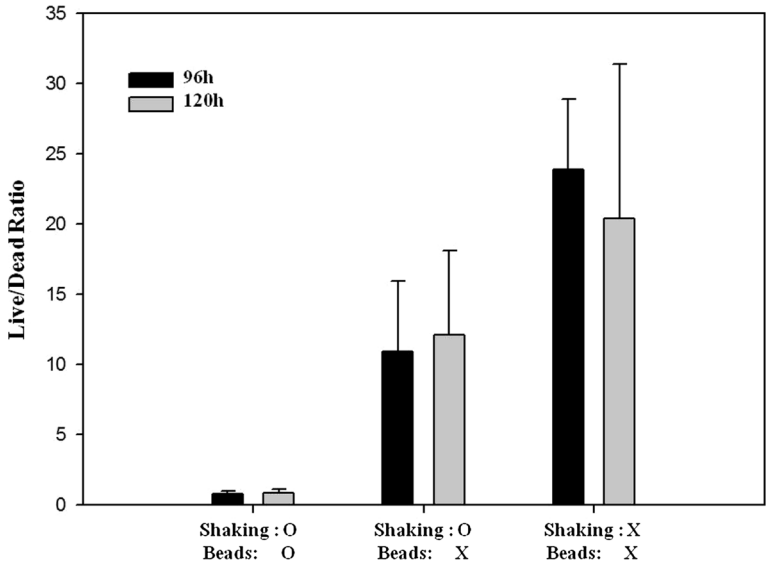

Fig. 2 The fluorescence ratio of live/dead cells. The ratio of live cells in the culture shaken with beads drastically decreased by shaking with beads. The graphs represent the average values from the biological replicate experiments (n=2)

as shaking with glass beads negatively affected the cell viability. In BG11, the proportion of live cells was larger in the condition without glass beads than that of shaking with glass beads. We assumed that the percentage of live/dead ratio was increased in the condition without physical stress due to small number of dying cells in BG11 (Fig. 2); however, the increase of live/dead ratio is more dramatic in BG11 than M145 (data not shown). The fluorescent images observed using a fluorescence microscope also show a large ratio of live cells without shaking, and beads and the BG11 cultured with shaking and glass beads relatively showed a small portion of live cells and ruptured vegetative mycelium (Fig. 3). Therefore, the growth of BG11 was very slow in the liquid medium, particularly from the physical stress.

Abnormal Membrane of *ndgR* Mutant in GlcNAc/Asn Liquid Medium

Cell membrane responses against the external stress, and we assumed that the poor growth and weakness against the physical stress occurred from the defect of the cell membrane because of the deletion of *ndgR*. Therefore, we observed the cell membranes directly by TEM to see whether BG11 had really weak cell membranes compared to the wild type. In complex R5 liquid media, BG11 grew well like wild type [17], and we assumed that there were no differences between the wild type and BG11. As expected, M145 and BG11 did not show significant differences, and the cell size and lipid layer were quite similar to each other. This was similarly observed in the cell growth and antibiotic production (Fig. 4 top panels). To confirm the role of NdgR in the synthesis of a cell membrane, M145 and BG11 were cultured in liquid MM GlcNAc/ASN medium, and their effect on the cell membrane was studied using TEM. In liquid MM GlcNAc/ASN, like wild type, BG11 showed vegetative hyphae with branches, crosswalls, and vegetative septa. However, BG11 had abnormal cell membranes. It was difficult to find the double-layered lipid membranes in BG11 compared to the wild type, and the amount and the size of cells were also much smaller than the wild type (Fig. 4 bottom panels). From

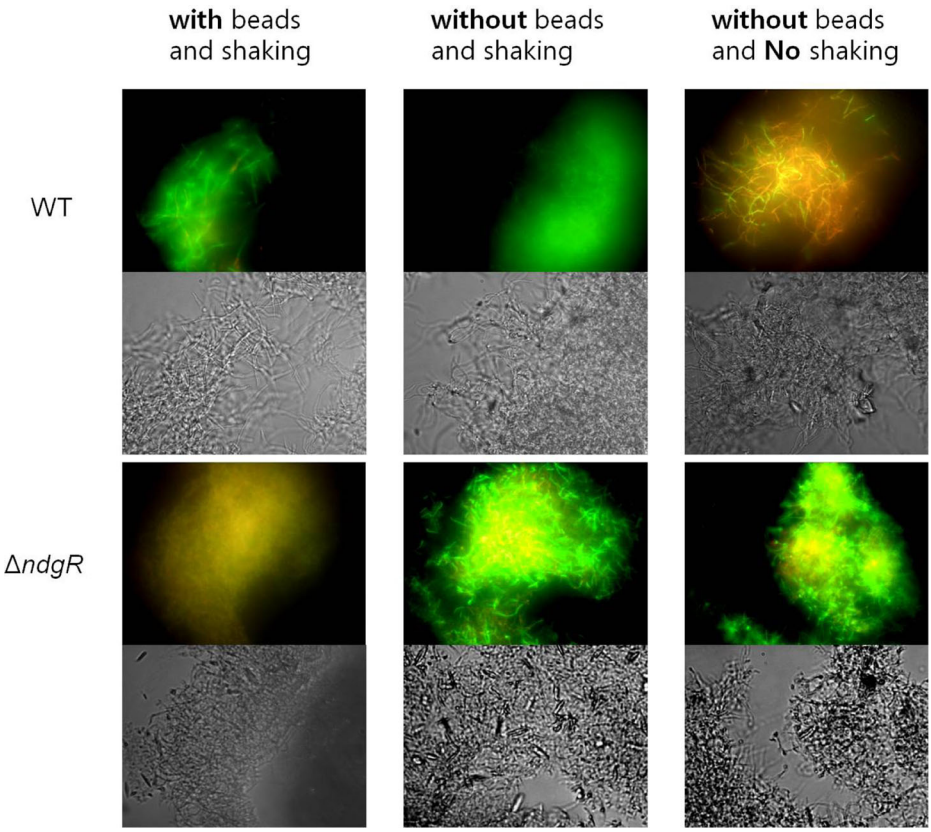

Fig. 3 Fluorescence images of live cells at 48 h (×1000). BG11 cultured with beads and shaking contains few live cells compared to other conditions

those results, we could infer that NdgR affects the cell membrane formation for tolerating the external physical stress. When phospholipid fatty acid compositions, the components of the membrane, between M145 and BG11 were compared in R5 and minimal media grown on a solid plate lined with cellulose sheet [8], the overall composition and lipid contents are quite similar to that in R5 (Fig. 5a, b). However, M145 and BG11 grown in minimal media were compared, and M145 contained more fatty acids such as 12-methyltetradecanoate and 15-methylhexadecanoate analyzed by GC-MS, indicating that BG11 has some different membrane structure or components from M145, which may be another reason to vulnerability of BG11. The exact detailed regulatory mechanism of NdgR is not known yet; however, the observation shows that NdgR certainly affected the formation of cell membrane directly and indirectly.

Discussion

The function of a regulator is very difficult to find because one regulator, particularly the global regulator, affects many parts including morphology, antibiotics, and growth in various nutrient conditions. Although, we reported that NdgR was associated with the antibiotic production, morphological change, and quorum sensing at the transcriptional level [17], there

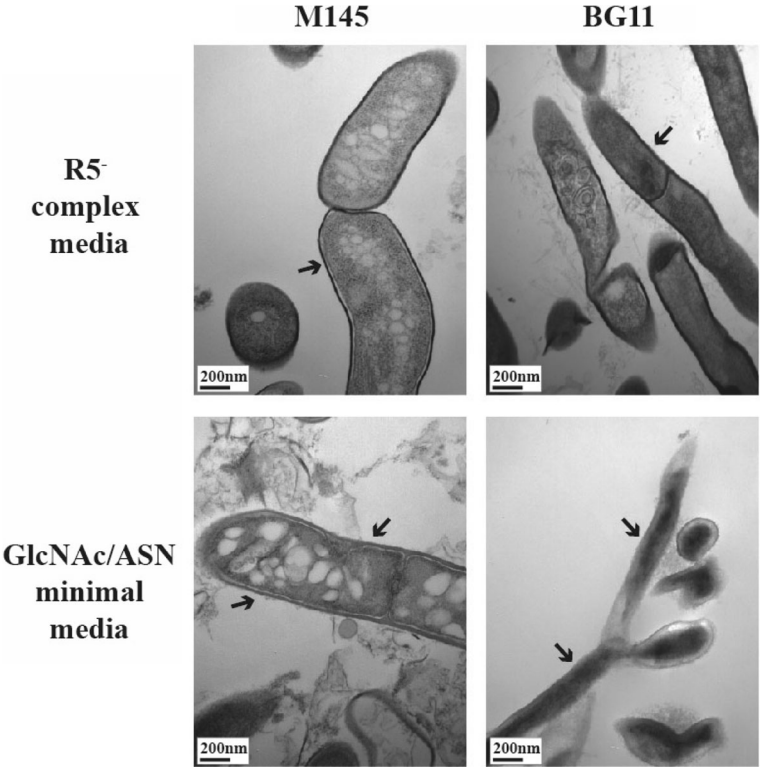

Fig. 4 TEM images (magnification, ×80 K) of wild type and BG11 grown in liquid R5 complex media and liquid MM GlcNAc/ASN. The cell membranes are indicated by *arrows*. The severe defects of lipid membranes were observed in BG11 cultured in liquid MM GlcNAc/ASN (*bottom right panel*)

are still many other functions. Although it was named as NdgR, nitrogen-dependent growth regulator, interesting evidences were found showing that its function can go beyond growth of the related regulator. In this study, we specifically found out another function of NdgR from comparatively observing the extremely poor growth (~2 % of M145) in liquid minimal medium to solid minimal medium. In addition to poor growth, BG11 was sensitive to the physical stress in liquid culture condition because of the poor structure of the cell membrane in *ndgR* mutant. In *ndgR* mutant, the deletion of *ndgR* may cause the lower expression of peptidoglycan precursors synthesizing genes, resulting in weak cell membrane from poor supply of building blocks. This could be even more clearly monitored in GlcNAc medium, because it is a direct precursor of peptidoglycan. Different utilization of *N*-acetylglucosamine affecting antibiotic production in BG11 were already reported [11], and these results were confirmed by lower mRNA expression level of *N*-acetylglucosamie-1-phosphate uridylyltransferase/glucosamine-1-phosphateacetyltransferase (*glmU*) and phosphoglucosaminemutase (*glmM*) in BG11 than M145 grown on *N*-acetylglucosamine, which are the major pathways to make peptidoglycan (data not shown). Moreover, NdgR is reported to positively affect *leuC* [12], which is the main enzyme for the synthesis of branched chain amino acids (BCAA) [1, 14, 15]. BCAA is one of the important precursors for the fatty acid synthesis, particularly for 12-methyl tetradecanoate and 15-methyl hexadecanoate [13]. Therefore, the deletion of *ndgR* caused poor supply of BCAA resulting in less production of fatty acids for cell membrane shown by GC-MS analysis (Fig. 5c, d). This weak structure was

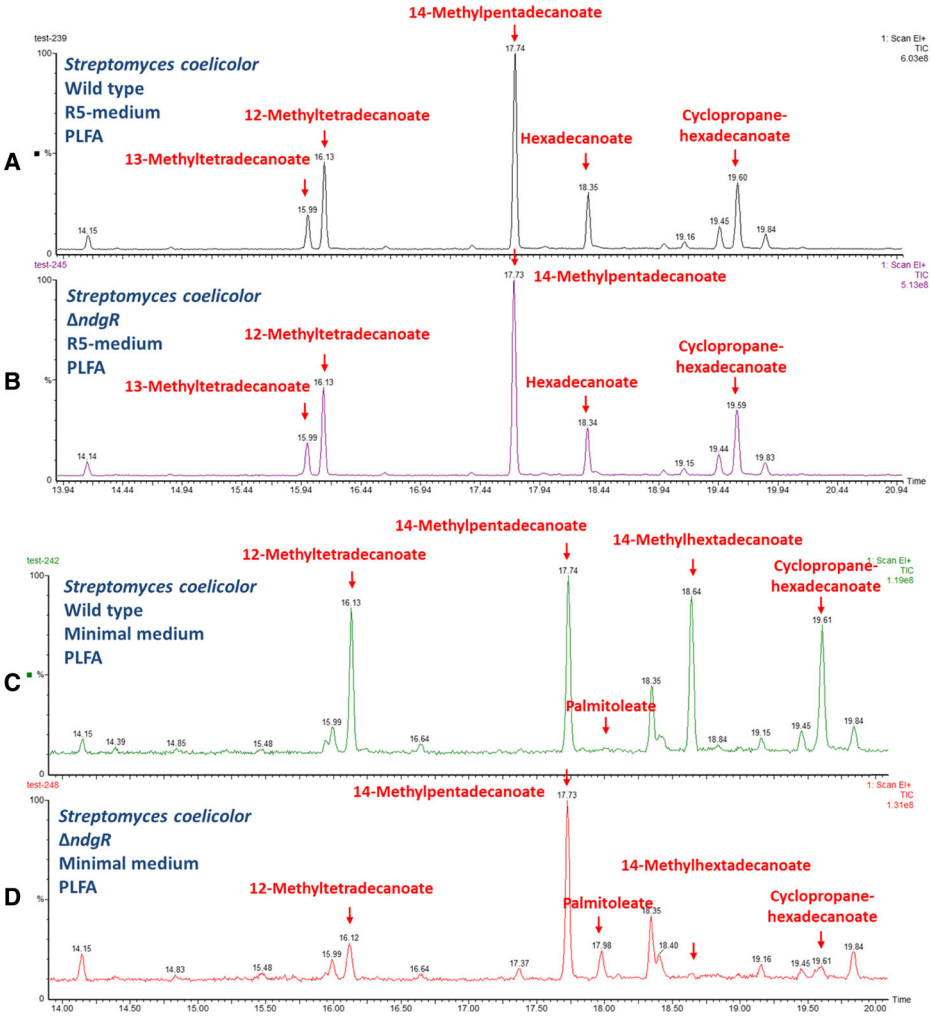

Fig. 5 GC-MS analysis of total lipids from M145 and BG11 cultured on cellulose membrane on minimal media plate after 4 days. Lyophilized cells were derivatized by fatty acid methyl ester with 15 % of H_2SO_4 in MeOH for 2 h. **a** and **b** were grown on R5, and **c** and **d** are grown on minimal media. The decreases of lipids such as 12-methyl tetradecanoate and 15-methyl hexadecanoate in BG11 with minimal media were observed

then vulnerable to the physical stresses from normal culture conditions with shaking and glass beads and led to a very low level of cell viability. Compared to shaking and glass bead culture, static culture of BG11 without shaking and bead showed better growth and more branches, which are the opposite to that in M145. Its cell mass, however, did not increase to wild-type level showing that there are also effects from nutrients on the growth by deletion of *ndgR*, which was monitored by solid minimal medium. While there may be effect from the chaperon and heat shock proteins [17], the synthesis of the cell membrane components like peptidoglycan and phopholipid is significantly affected by the deletion of *ndgR* due to weak cell membrane with high sensitivity under normal culture condition. Therefore, the function of NdgR seems more global than expected.

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