Cell membrane and electron transfer engineering for improved synthesis of menaquinone-7 in *Bacillus subtilis*

Shixiu Cui, Hongzhi Xia, Taichi Chen, Yang Gu, Xueqin Lv, Yanfeng Liu, Jianghua Li, Guocheng Du, Long Liu

PII: S2589-0042(20)30102-4
DOI: https://doi.org/10.1016/j.isci.2020.100918
Reference: ISCI 100918

To appear in: *iScience*

Received Date: 4 November 2019
Revised Date: 9 January 2020
Accepted Date: 11 February 2020

Please cite this article as: Cui, S., Xia, H., Chen, T., Gu, Y., Lv, X., Liu, Y., Li, J., Du, G., Liu, L., Cell membrane and electron transfer engineering for improved synthesis of menaquinone-7 in *Bacillus subtilis*, *iScience* (2020), doi: https://doi.org/10.1016/j.isci.2020.100918.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 The Author(s).
Cell membrane and electron transfer engineering for improved synthesis of menaquinone-7 in *Bacillus subtilis*

Shixiu Cui$^{1,2}$#, Hongzhi Xia$^{3}$#, Taichi Chen$^{1,2}$, Yang Gu$^{1,2}$, Xueqin Lv$^{1,2}$, Yanfeng Liu$^{1,2}$, Jianghua Li$^{1,2}$, Guocheng Du$^{1,2}$, and Long Liu$^{1,2,4,*}$

$^1$Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China

$^2$Key Laboratory of industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China

$^3$Richen Bioengineering Co., Ltd, Nantong 226000, China

$#$These authors contributed equally

$^4$Lead Contact

*Correspondence: longliu@jiangnan.edu.cn
Summary

The formation of biofilm facilitates the synthesis of valuable natural product Menaquinone-7 (MK-7) in static culture of *Bacillus subtilis*, while the essential role and mechanism of biofilm in MK-7 synthesis have not been revealed. Herein, comparative transcriptomics show that the formation of biofilm affected MK-7 synthesis by changing the transcription levels of signal receptor (BSU02010), transmembrane transporter (BSU29340, BSU03070) and signal transduction (BSU02630). Moreover, we also found that oxalate decarboxylase OxdC has an important effect on electron generation and MK-7 synthesis, when the transcriptional level of NADH dehydrogenase decreases in static culture. Our results revealed that cell membrane and electron transfer are important factors in promoting MK-7 synthesis.

Introduction

As a highly valuable vitamin K2, Menaquinone-7 (MK-7) is a polyene compound consisting of 2-methyl 1,4-naphthoquinones ring structure with a side chain of 7 units isoprene (Berenjian *et al*., 2015). It was reported that MK-7 is the component of microbial plasma membrane and plays an important role in electron transport and oxidative phosphorylation (Berenjian *et al*., 2013). Owing to the good bioavailability, MK-7 functions in protecting human health (Sato *et al*., 2012), such as prevention of osteoporosis (Iwamoto, 2014), arterial calcification, cardiovascular and parkinsons diseases (Grober *et al*., 2014; Ravishankar *et al*., 2015).

In the past decades, many studies have focused on improving the production of MK-7 by microbial fermentation (Berenjian *et al*., 2011; Mahanama *et al*., 2011; Wu and Ahn, 2011). For example, by optimizing medium nutrients the maximum concentration of menaquinone-7 reached 62.32 mg/L (Berenjian *et al*., 2011), the fed-batch culture modes were then adopted to increase the titer of MK-7 by 40% to 86.48 mg/L (Berenjian
et al., 2012). Furthermore, Yang et al. (2019) divided the MK-7 synthesis pathways into four modules and overexpressed several key genes including menA, dxs, dxr, yacM-yacN, glpD, resulting in the increase of MK-7 titer to 69.5 mg/L. It was noteworthy that static culture was likely to be beneficial to MK-7 production, which may be attributed to the biofilm formation in static culture (Berenjian et al., 2013). As well known, biofilm is an assemblage of tightly associated bacteria encapsulated by a self-produced extracellular matrix (Mielich-Suss and Lopez, 2015), and creates favorable conditions for sustainable survival in the natural environment (Cairns et al., 2014). Interestingly, Mahdinia et al. (2018) used the biofilm reactor with four different types of plastic composite carriers to produce MK-7, and increased the MK-7 titer by 2.3-fold to 28.7 mg/L. However, the specific role and mechanism of biofilm in the synthesis of MK-7 have not been revealed at genetic level and this limits the possibility of further increasing MK-7 production.

To illuminate the intrinsic connections between the biofilm formation and MK-7 production, Bacillus subtilis, a Gram-positive model microorganism which has been widely used in the production of nutraceuticals and enzymes (Song et al., 2015), was selected as the host in this work. First, by knocking out the biofilm-forming genes epsA-C, tasA, sinl, yuaB and ftsH, we verified that biofilms have significant effects on MK-7 synthesis. Then, the comparative transcriptomics analysis of the strains in shake culture and static culture was performed and the results showed that the expression of genes involved in biological process, molecular function and cellular component was up-regulated. In particular, some differential genes were related to the membrane components, such as transmembrane transporter (BSU29340, BSU03070), and the expression level of eight NADH dehydrogenases was down-regulated. Moreover, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and NADH content analysis showed that the oxalate-decarboxylase (OxdC) was abundantly expressed and could provide a large number of electrons through the delivery of cytochrome C and
MK-7 in static culture. Finally, combinatorial overexpression of cell membrane protein tatAD-CD and menaquinol-cytochrome C reductase qcrA-C significantly increased the titer of MK-7 from 200 to 310 mg/L in a 15-L bioreactor. All these results demonstrate that biofilm can promote the synthesis of MK-7 of B. subtilis by modulating the cell membrane components and electron transfer.

RESULTS

Biofilm significantly affects the synthesis of MK-7 in B. subtilis

Biofilm is a dynamic community composed of at least three cell types including matrix-producing cells, motile cells and sporulation cells (Cairns et al., 2014). These cells are encapsulated by the matrixes including exopolysaccharides (EPS), fibrils formed by secreted protein (TasA), and extracellular DNA (Figure 1). In B. subtilis, the EPS and TasA are encoded by the epsA-C operon and yqxM-tasA operon, respectively (Romero et al., 2010; Diehl et al., 2018). As shown in Figure 2A, deletion of epsA-C and yqxM-tasA operons inhibited the formation of B. subtilis biofilm in static culture, resulting in a significant decrease of specific MK-7 titer from 1.6 µg/mg of B. subtilis 168 to 1.2 µg/mg and 0.65 µg/mg, respectively. Compared with the B. subtilis 168, the biomass of cells with deletion of epsA-C and yqxM-tasA operons increased by 44% and 35%, respectively (Figure 2B). Considering that the expression of yqxM-tasA operons is controlled by the regulatory repressor SinI and anti-repressor protein SinR (Branda et al., 2006; Chu et al., 2006), we deleted sinI gene and found that the specific MK-7 titer decreased from 1.60 µg/mg to 0.46 µg/mg and the biomass increased from 14.3 g/L to 17.5 g/L (Figure 2A, B). Moreover, YuaB, the major proteinaceous component of matrix, is essential to form a water-repellent surface layer in the biofilm by self-assembly (Kobayashi and Iwano, 2012; Liu et al., 2017). Knockout of the gene yuaB inhibited the formation of biofilm and the specific MK-7 titer decreased by 37.5% to 1 µg/mg (Figure 2A, B). Besides, the membrane-bound protease FtsH is also important for biofilm formation.
(Yepes et al., 2012), and here the deletion of ftsH gene decreased the specific MK-7 titer from 1.60 µg/mg to 0.69 µg/mg (Figure 2A, B). In addition, the combinatorial deletion of these genes significantly decreased the biofilm formation and MK-7 synthesis (Figure 2A). In particular, when yqxA-tasA operon and yuaB were knocked out simultaneously, the synthesis of extracellular polysaccharide and protein was blocked and almost no biofilm was formed (Figure 2A), resulting in a significant decrease of specific MK-7 titer to only 0.25 µg/mg (Figure 2C), while little influence on cell growth (Figure S1). Simultaneous deletion of tasA and yuaB genes may change the mechanical strength of the matrix, resulting in a more homogenous pattern of cell death. On the other hand, disassembly of bacterial biofilm has a negative effect on the attachment of bacterial cell wall and extracellular matrix to affect cell growth (Mielich-Suss and Lopez, 2015). The above results indicate that biofilm formation was related with MK-7 synthesis and the inhibition of biofilm formation can significantly reduce MK-7 synthesis.

Comparative transcriptomics analysis of B. subtilis strains in static and shake culture

The comparative transcriptomics analysis was performed to analyze the changes of global gene expression in MK-7 synthesis by static culture and shake culture. The transcriptomics data have been deposited in the NCBI Sequence Read Archive (SRA) (accession number: PRJNA599448). Table S1 shows the statistical summary of the transcriptome sequencing data, and the data from all differentially expressed genes in B. subtilis were used for systematic cluster analysis to gain insight into the differences between the transcriptomes of static and shake culture. The heat map suggests that the selected differential genes are clearly consistent (Figure S2).

The comparative transcriptomics results show that the expression of most genes in glycolysis pathway, pentose phosphate pathway and shikimic acid pathway was up-regulated in static culture, while the expression...
of genes in 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway and MK-7 synthesis pathway was
down-regulated in static culture (Figure 3A). The quantitative reverse transcription polymerase chain reaction
(qRT-PCR) was also used to verify the result of comparative transcriptomics analysis, and both results were
consistent (Figure S3). That is to say, static culture could up-regulate the central metabolic pathway genes and
enhance the chorismic acid synthesis. In particular, compared to the shake culture, the expression levels of
most NADH dehydrogenases except glpD, yumB and ahpF significantly decreased in the static culture (Figure
3B). Further investigation of the expression of the other dehydrogenases showed that the expression of nearly
half of the dehydrogenases was up-regulated (Figure 3C). In addition, the expression levels of various amino
acid dehydrogenases such as ycgM, gcvpA, dhaS, bcd, ald, asd, hisD, tyrA and gudB increased. These results
indicate that the central metabolic pathways and amino acid metabolism were promoted in static culture.

To systematically analyze the effect of differential genes on MK-7 synthesis, we classified them by Volcano
map and Veen diagram and a total of 2093 differentially expressed genes were obtained (Figure 4A).
Compared with the shake culture, the expression levels of 1281 genes were up-regulated (Table S2) and 812
genes were down-regulated (Table S3) in static culture. As shown in Figure 4B, 3919, 3973, 3586, and 3581
transcripts were detected in BS168 static-1, BS168 static-2, BS168 shake-1 and BS168 shake -2, respectively.
There were 3345 transcripts common to all the samples. Filtering analysis, Gene Ontology (GO) functional
annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were carried out and
the results show that GO functional annotation classified the differential genes into three categories: molecular
function, cellular component and biological process, while KEGG pathway analysis classified the differential
genes into six categories with the most significant difference in metabolism category (Figure 4C, D). To
determine the gene functional classes in static culture, we carried out a homology-based annotation
specifically for all the 2093 differentially expressed genes, and GO terms related to the data set are shown in Table S4. Significant differences were found in expression of genes involved in biological process, molecular function and cellular component (Figure 4C). In particular, the changes were obviously in the membrane component (GO:0006810, GO:0017000, GO:0008125, GO:0031224, GO:0051234), indicating that cell membrane could affect MK-7 synthesis. Moreover, the enrichment of terms related to biotic stress and oxidative stress were distinctly (GO:0043207, GO:0006979). In addition, enrichment of gene functions related to transporter and transcription were observed (GO:0071944, GO:0051234, GO:0006139), which possibly generated a response of the external stimuli and transmit signals into cells to regulate gene expression. In general, compared to shake culture, the enrichment analysis of GO terms indicates that the differential genes were mainly related to signal transduction and membrane components (Figure 4C), indicating that the unknown signals transduction generated in static culture may lead to changes in membrane components to regulate the synthesis of MK-7.

The set of 2093 differentially expressed genes was mapped onto KEGG pathways in \textit{B. subtilis} 168 (Table S5), highlighting the involvement of amino acid, cofactors and vitamin metabolism as well as the transmembrane transport. Amino acid metabolism in static culture was significantly different from that in shake culture. The transcripts of synthetases involved in amino acids were up-regulated, except tryptophan and threonine dehydrogenase, indicating that amino acid synthesis may affect the synthesis of MK-7. Two other important pathways, “Two component system” (ko02020) and “ABC transporters” (ko02010), were also found to be up-regulated in static culture (Figure 4D). PhoD (alkaline phosphatase D), alkaline phosphatase synthesis sensor protein PhoR and phosphate transport system substrate-binding protein PstS, associated with the transfer of phosphate groups were all up-regulated in static culture. Additionally, several transcripts from \textit{ydfI}
(transcriptional regulator), *phoAB* (alkaline phosphatase), *cheY* (chemotaxis protein), *ssuA* (sulfonate transport system substrate-binding protein), *mntB* (manganese/zinc/iron transport system ATP-binding protein), *cysC* (adenylyl sulfate kinase), and *ytrB* (acetoin utilization transport system ATP-binding protein) were also up-regulated in static culture. All these genes have been reported to be involved in response to several stresses, such as phosphate limitation (Antelmann *et al.*, 2000) and metal ion stress (Que and Helmann, 2000). The synthesis of secondary metabolites (ko01110) also had significant differences, including coenzymes and vitamins, such as Biotin (ko00780), thiamine (ko00730) and folate (ko00790). Taken together, these results indicate that compared with shake culture, static culture had significant effects on signal transduction, amino acid metabolism, and secondary metabolite synthesis.

**Overexpression of differential genes in *B. subtilis* 168 and the engineered strain BS20**

According to the above analysis, influence of the differential genes on the synthesis of MK-7 by the static culture of *B. subtilis* 168 was first examined. Table S6 shows the selected differential genes, which are involved in signal transduction, transmembrane transport, and oxidative phosphorylation. According to the influence of differential genes overexpression on biofilm formation, these genes can be divided into two groups: promoting biofilm formation group and inhibiting biofilm formation group. The former group mainly includes the genes BSU30760, BSU29340, BSU34265, BSU03070, BSU02010 and BSU02630. When these genes were overexpressed by the strong constitutive promoter $P_{43}$, the cells concentrated on the surface of medium, and a large amount of biofilm formed compared to *B. subtilis* 168 (Figure 5A). The specific MK-7 titer was increased by 45% and 160% when BSU02630 and BSU030760 were overexpressed in *B. subtilis* 168, respectively. Especially when overexpressing BSU03070, the specific MK-7 titer was increased to 6.0 µg/mg, 3.75-fold that of *B. subtilis* 168 (Figure 5B). In addition, there were significant differences in biofilm morphology among
strains overexpressing BSU30760, BSU29340, BSU34265, BSU03070, BSU02010 and BSU02630. The surface of the biofilm in the strains overexpressing BSU30760 or BSU03070 was smooth without wrinkles, while the overexpression of other genes generated a large number of wrinkles. Strangely, the biofilm formed by BSU34265 overexpression was not as smooth as the other biofilms, and a large number of particles appeared on the surface. However, the cells overexpressing BSU08840, BSU02000, BSU01910, BSU24640, BSU03200 and BSU05410 settled at the bottom of the medium without biofilm formation (Figure 5A), and the specific MK-7 titer decreased from 1.6 $\mu$g/mg to 0.5-1.45 $\mu$g/mg. Moreover, overexpression of these genes except BSU24640 and BSU02000 significantly decreased the biomass (Figure 5B). Finally, the constitutive strong promoter $P_{43}$ was used for overexpression of the genes that can promote biofilm formation and we investigated 15 arrays containing different combinations of genes (Figure S4). Figure 5C shows that the titer of MK-7 did not further increase and for co-overexpression of BSU29340 and BSU34265, the biomass of cells decreased from 14.3 to 10 g/L (Figure S5).

In a recent work, we have developed a bifunctional quorum-sensing system in *B. subtilis* 168 to engineer the synthesis modules of MK-7, and obtained a recombinant strain BS20, which can produce 360 mg/L MK-7 in shaker flask and 200 mg/L MK-7 in 15-L bioreactor (Cui *et al.*, 2019). In this work, we further verified the influence of differential genes on MK-7 synthesis in the engineered strain BS20 in the static culture. It was found that different from *B. subtilis* 168, the overexpression of differential genes BSU13610, BSU08840, BSU29430, BSU02000, BSU34265, BSU36660, BSU03070, BSU02010, BSU02630, BSU03200 and BSU05410 in BS20 can promote to form a large amount of biofilms (Figure 5D), and the biofilms are more wrinkled. Moreover, the specific MK-7 titer of the strains overexpressing the above genes was significantly improved compared to BS20, with an increase range of 20-400% (Figure 5E). Specifically, the synthesis of
MK-7 increased from 9.02 to 42.5 µg/mg in the strain overexpressing BSU02000. In addition, compared with the biomass of *B. subtilis* 168, the biomass of strain BS20 increased by nearly 2-3 times and reached a maximum of 50 g/L (Figure 5B, E). Finally, the specific MK-7 titer was increased by 30-50% by overexpression of differential genes in combination compared with that in single gene overexpression. For example, in the strains co-expressing BSU34265 and BSU02630 or co-expressing BSU29340 and BSU03070, the specific MK-7 titer increased from 9.02 to 65 µg/mg and 58 µg/mg, respectively (Figure 5F). In summary, the signal transduction (BSU02000, BSU02630) and transmembrane transport (BSU34265, BSU29340, BSU03070) in the strain BS20 had significant effects on the synthesis of MK-7.

**Electron transfer has significant effects on MK-7 synthesis in *B. subtilis***

MK-7 plays an important role in electrons transport, oxidative phosphorylation and sporulation (Berenjian *et al.*, 2013). NADH is a molecule for donating electrons. Electrons are transported by MK-7 and cytochrome C, and finally oxygen acts as an electron acceptor to form water (Figure 6A). Besides, the cytochromes in *B. subtilis* 168 mainly include *ctaC-G* operator and *qcrA-C* operator (Yu *et al.*, 1995; Lyons *et al.*, 2012; Kolodkin-Gal *et al.*, 2013; Qin *et al.*, 2019). The expression level of *catC-G* and *qcrA-C* were up-regulated in static culture compared with that in shake culture (Figure S6). Moreover, to further verify the effect of cytochrome on MK-7 synthesis, we increased the expression level of menaquinol-cytochrome c reductase QcrA-C by the constitutive promoter *P*43 in *B. subtilis* 168 and BS20, respectively, and found that there was a significant difference in biofilm morphology between the two strains. The overexpression of *qcrA-C* in *B. subtilis* 168 made the biofilm look smooth with a few wrinkles, and the specific MK-7 titer increased from 1.6 to 4.0 µg/mg, while the *qcrA-C* overexpression in BS20 made the biofilm form obvious wrinkles (Figure 6B), and the specific MK-7 titer increased from 9.02 to 28.0 µg/mg (Figure 6C). In addition, it was found that increasing the copy number of
qcrA-C to 2 in BS20 can further improve the synthesis of MK-7 to 62.3 µg/mg, 2.23-fold that of single copy (Figure 6C). These results show that the increased expression of menaquinol-cytochrome reductase could significantly promote the synthesis of MK-7, indicating that more MK-7 can deliver more electrons, and MK-7 plays an indispensable role in electron transfer.

As mentioned above, NADH donates the electrons under action of NADH dehydrogenase, and transfers electrons to the electron transport system (ETM) and pumps protons out of the cell. However, the expression of most NADH dehydrogenases was down-regulated. Therefore, we speculate that there may be other processes that can donate large amounts of electrons. In order to confirm the above hypothesis, we analyzed the cellular proteins and found a very distinct band at 46 KDa using SDS-PAGE (Figure 6D). Combined with protein flight mass spectrometry, we revealed that the band was oxalate-decarboxylase (OxdC) (Figure S7), which requires Mn and O₂ to catalyze the conversion of oxalate to formate and CO₂(Conter et al., 2019), and then formate is oxidized to CO2 and electrons by formate dehydratase (FdhD and YrhE) (Wilks et al., 2009, Glaser et al., 1995, Yang et al., 2015). Figure 6D shows that the expression of OxdC increases with the fermentation time. Next, we investigated the effect of deleting gene oxdC and blocking electron transport on the biofilm morphology and MK-7 synthesis in static culture. It was found that the knockout of oxdC in BS20 causes obvious changes in the morphology of biofilm. As shown in Figure 6E, the wrinkles of biofilm formed by the oxdC deletion strain BS20-ΔoxdC were less than that of BS20, while the biomass of BS20-ΔoxdC increased from 27.3 to 31.0 g/L (Figure 6F). The specific MK-7 titer also increased with fermentation time, and the specific MK-7 titer of BS20-ΔoxdC reached 17.5 µg/mg on the fourth day, 1.94-fold that of BS20 (Figure 6F). In addition, when oxdC was knocked out, the expression of NADH dehydrogenase ldH, bdhA, ndH and sdhA-C was up-regulated (Figure 6G), indicating that after oxdC knockout, NADH dehydrogenase compensates for the absence of
electrons. These results indicate that the stability of electron transfer chain could increase the synthesis of MK-7. Moreover, the ratio of NADH/NAD$^+$ can characterize the rate of intracellular electrons extracted, and the NADH level in BS20 was higher than that of BS20-$\Delta$oxdC, reaching 1.2 nmol/g on the second day, while the level of NAD$^+$ in BS20-$\Delta$oxdC was higher than that of BS20 (Figure 6H), indicating that stable electron supply and transfer are important for the efficient synthesis of MK-7.

**MK-7 production by the engineered *B. subtilis* strain in 250 mL flask and 15-L bioreactor**

Considering that signal transduction protein TatAD-CD (BSU02630) and methylphenol cytochrome c reductase QcrA-C can significantly promote the synthesis of MK-7, we co-expressed the *tatAD-CD* (BSU02630) and *qcrA-C* using $P_{\alpha\alpha}$ promoter in the engineered strain BS20 to study the effect of signal transduction and electron transfer on MK-7 synthesis in shake culture. Figure 7A shows that the MK-7 titer of strains BS20-T (*tatAD-CD* overexpression) and BS20-Q (*qcrA-C* overexpression) increased from 360 to 370 mg/L and 375 mg/L, respectively. The cell growth of BS20-T was increased and the maximum OD$_{600}$ was 127% of BS20 (Figure 7B).

To detect the substrate conversion rate, we detected the glucose content in static culture and shake culture. The results show that the consumption of glucose in shake culture was significantly higher than that in static culture (Figure S8A), and the MK-7 yield on glucose of the strains BS20-T and BS20-Q were 0.86 mg/g and 0.83 mg/g, respectively. In addition, after 2.5 days of fermentation, the cells hardly consumed glucose in static culture, while glucose was consumed in shaking culture (Figure S8B). Moreover, the co-overexpression of *tatAD-CD* and *qcrA-C* in BS20 using $P_{\alpha\alpha}$ promoter, yielding the engineered strain BS20-QT, increased the titer of MK-7 from 360 to 410 mg/L in shake culture. We performed SDS-PAGE on whole cell proteins of strains BS20-Q and BS20-T in shaking culture to analyze the protein abundance of the over-expression targets. The result shows that the expression of QcrA (18.74 kDa), QcrB (25.94 kDa) and QcrC (28.17 kDa) was increased
compared with BS20. However, the expression of TatAD (7.43 kDa) and TatCD (27.71 kDa) did not change obviously (Figure S10), maybe due to the fact that TatAD and TatCD are membrane proteins.

Based on the above results, the engineered strain BS20-QT was further cultured in a 15-L fed-batch bioreactor for MK-7 production. In fed-batch cultivation with control of glucose concentration between 8-16 g/L, a total of 800 mL of feeding solution was added to the bioreactor during the culture period. As shown in Figure 7C, compared with the strain BS20, the MK-7 titer of BS20-QT was increased from 200 to 245 mg/L under the condition of low dissolved oxygen level (30%). Under the condition of high dissolved oxygen level (55%), the maximum MK-7 titer of BS20-QT in a 15-L bioreactor increased from 200 to 310 mg/L, and the maximum OD$_{600}$ of BS20-QT was increased by 14.3% than that under the low dissolved oxygen (Figure 7D). In the early stage of fermentation, especially during the period of cell exponential growth, the dissolved oxygen level in the fermentation broth was only 5-8% (Figure S9). These results demonstrated that fed-batch cultivation with control of high dissolved oxygen level was favorable for MK-7 production and provided a basis for large-scale production of MK-7.

DISCUSSION

In *B. subtilis*, the robust biofilm formation requires large quantities of ferric iron to promote the production of iron-containing enzymes involved in respiratory electron transfer, and establishes strong membrane potential, which is considered to be a key factor in the production of biofilm matrix (Qin *et al.*, 2019). Fang *et al.* (2018) proved that the cobalt uptake transport system is essential for *de novo* vitamin B12 biosynthesis in *Escherichia coli*. Furthermore, MK-7 is a component of bacterial cell membranes and plays an important role in electron transfer, and thus, the state of cell membrane may also affect the synthesis of MK-7. Wang *et al.* (2019) detected the relationship between the concentration of MK-7 and the value of surface tension and found that...
maintaining the stable surface tension of cell membrane is beneficial to the accumulation of MK-7.

Ranmadugala et al. (2017) discovered that changing the state or composition of the membrane in *B. subtilis natto* could increase the synthesis of MK-7. Herein, we found that the expression level of cell membrane-associated proteins changed most significantly under shake culture and static culture, and further overexpressing these membrane protein genes could promote the synthesis of MK-7. These results indicate that cell membranes play a significant role in the synthesis of MK-7, which may be owing to the fact that stable state and composition of cell membrane will provide a stable synthesis and storage platform for MK7, and in return, high concentration of MK7 could ensure the efficiency of electron transfer.

OxdC can catalyze the conversion of oxalate to formate and CO$_2$, and then extracts electrons from formate oxidation (Yang et al., 2015). The protein content of OxdC was significantly higher in static culture, which was important for electron generation. Knockout of *oxdC* did not reduce the production of MK-7, which may be because the increased expression level of NADH dehydrogenase (Figure.6G) also could maintain the concentration of electron to stabilize the efficiency of electron transfer. Previous studies showed that the concentration of MK-7 was inversely proportional to the ratio of NADH/NAD$^+$ in *B. subtilis* (Wang et al., 2019). It was reported that the synthesis of coenzyme Q (transport electrons like MK-7) was promoted by increasing the NADH/NAD$^+$ ratio in *Rhodobacter sphaeroides* (Zhu et al., 2017). The reason may be that as a Gram-positive aerobic bacterium, *B. subtilis* can only use MK-7 as the carrier of electron transport, while *E. coli* and *R. sphaeroides* are facultative anaerobes, which can utilize ubiquinone (CoQ-8) to transport electrons under aerobic conditions and use MK-7 when grow anaerobically. It was reported that increasing the supply of oxygen could effectively improve the synthesis of MK-7 (Berenjian et al., 2014). In this study, overexpression of *qcrA-C* increased the efficiency of electron transport to oxygen and increasing the supply of oxygen in the 15-L
bioreactor was beneficial to the synthesis of MK-7. These results also confirmed that the sufficient electron transport was an important factor for efficient synthesis of MK-7.

In summary, we demonstrated that biofilm has significant effects on the MK-7 synthesis by comparative transcriptomics analysis of the strains in shake culture and static culture. Besides, we clarified that the electron transport also has remarkable influence on the synthesis of MK-7. The co-overexpression of the cell membrane components signals transduction protein tatAD-CD (BSU02630) and menaquinol-cytochrome c reductase qcrA-C in BS20 increased the MK-7 titer in 15-L bioreactor by 55% to 310 mg/L.

Limitations of the Study

Here we show the effect of cell membrane-associated proteins and electron transport on MK-7 synthesis. The synthesis of MK-7 is also affected by temperature and pH, and the corresponding regulatory transcription factors have not been explored in this study. Further work will focus on the regulation of global transcription factors related to temperature and pH to further promote the synthesis of MK-7.

Methods

All methods can be found in the accompanying Transparent Methods supplemental file.

Acknowledgments

This work was financially supported by the Key Research and Development Program of China (2018YFA0900300, 2018YFA0900504), the National Natural Science Foundation of China (31622001, 31871784, 31870069, 31671845, 31930085), the Fundamental Research Funds for the Central Universities (JUSRP51713B), and Postgraduate Research & Practice Innovation Program of Jiangsu Province.
Author Contributions
L.L., L.J., G.D. designed the research. S.C., H.X. and T.C. performed the experiments. L.L., S.C., Y.G., Y.L. and X.L. analyzed the data. L.L., S.C., Y.G., X.L. wrote the manuscript.

Declaration of Interests
The authors declare no competing interests.

References
Antelmann, H., C. Scharf and M. Hecker, 2000. Phosphate starvation-inducible proteins of \textit{Bacillus subtilis}: Proteomics and transcriptional analysis. J Bacteriol, 182(16): 4478-4490.
Berenjian, A., N.L. Chan, R. Mahanama, A. Talbot, H. Regtop, J. Kavanagh and F. Dehghani, 2013. Effect of biofilm formation by \textit{Bacillus subtilis} natto on menaquinone-7 biosynthesis. Mol Biotechnol, 54(2): 371-378.
Berenjian, A., R. Mahanama, J. Kavanagh and F. Dehghani, 2015. Vitamin k series: Current status and future prospects. Crit Rev Biotechnol, 35(2): 199-208.
Berenjian, A., R. Mahanama, A. Talbot, R. Biffin, H. Regtop, P. Valtchev, J. Kavanagh and F. Dehghani, 2011. Efficient media for high menaquinone-7 production: Response surface methodology approach. N Biotechnol, 28(6): 665-672.
Berenjian, A., R. Mahanama, A. Talbot, H. Regtop, J. Kavanagh and F. Dehghani, 2012. Advances in menaquinone-7 production by \textit{Bacillus subtilis} natto: Fed-batch glycerol addition. American Journal of
Berenjian, A., R. Mahanama, A. Talbot, H. Regtop, J. Kavanagh and F. Dehghani, 2014. Designing of an intensification process for biosynthesis and recovery of menaquinone-7. Appl Biochem Biotechnol, 172(3): 1347-1357.

Branda, S.S., F. Chu, D.B. Kearns, R. Losick and R. Kolter, 2006. A major protein component of the Bacillus subtilis biofilm matrix. Mol Microbiol, 59(4): 1229-1238.

Cairns, L.S., L. Hobley and N.R. Stanley-Wall, 2014. Biofilm formation by Bacillus subtilis: New insights into regulatory strategies and assembly mechanisms. Mol Microbiol, 93(4): 587-598.

Chu, F., D.B. Kearns, S.S. Branda, R. Kolter and R. Losick, 2006. Targets of the master regulator of biofilm formation in Bacillus subtilis. Mol Microbiol, 59(4): 1216-1228.

Conter, C., E. Oppici, M. Dindo, L. Rossi, M. Magnani and B. Cellini, 2019. Biochemical properties and oxalate-degrading activity of oxalate decarboxylase from Bacillus subtilis at neutral ph. IUBMB Life, 71(7): 917-927.

Cui, S., X. Lv, Y. Wu, J. Li, G. Du, R. Ledesma-Amaro and L. Liu, 2019. Engineering a bifunctional phr60-rap60-spo0a quorum-sensing molecular switch for dynamic fine-tuning of menaquinone-7 synthesis in Bacillus subtilis. ACS Synth Biol, 8(8): 1826-1837.

Diehl, A., Y. Roske, L. Ball, A. Chowdhury, M. Hiller, N. Moliere, R. Kramer, D. Stoppler, C.L. Worth, B. Schlegel, M. Leidert, N. Cremer, N. Erdmann, D. Lopez, H. Stephanowitz, E. Krause, B.J. van Rossum, P. Schmieder, U. Heinemann, K. Turgay, U. Akbey and H. Oschkinat, 2018. Structural changes of tasa in biofilm formation of Bacillus subtilis. Proc Natl Acad Sci U S A, 115(13): 3237-3242.

Fang, H., D. Li, J. Kang, P. Jiang, J. Sun and D. Zhang, 2018. Metabolic engineering of Escherichia coli for de novo biosynthesis of vitamin B12. Nat Commun, 9(1): 4917.
Glaser, P., A. Danchin, F. Kunst, P. Zuber and M.M. Nakano, 1995. Identification and isolation of a gene required for nitrate assimilation and anaerobic growth of *Bacillus subtilis*. J Bacteriol, 177(4): 1112-1115.

Grober, U., J. Reichrath, M.F. Holick and K. Kisters, 2014. Vitamin k: An old vitamin in a new perspective. Dermatoendocrinol, 6(1): e968490.

Iwamoto, J., 2014. Vitamin k(2) therapy for postmenopausal osteoporosis. Nutrients, 6(5): 1971-1980.

Kobayashi, K. and M. Iwano, 2012. Bsla(yuab) forms a hydrophobic layer on the surface of *Bacillus subtilis* biofilms. Mol Microbiol, 85(1): 51-66.

Kolodkin-Gal, I., A.K. Elsholz, C. Muth, P.R. Girgis, R. Kolter and R. Losick, 2013. Respiration control of multicellularity in *Bacillus subtilis* by a complex of the cytochrome chain with a membrane-embedded histidine kinase. Genes Dev, 27(8): 887-899.

Liu, W., S. Li, Z. Wang, E.C.Y. Yan and R.M. Leblanc, 2017. Characterization of surface-active biofilm protein bsla in self-assembling langmuir monolayer at the air-water interface. Langmuir, 33(30): 7548-7555.

Lyons, J.A., D. Aragao, O. Slattery, A.V. Pisliakov, T. Soulimane and M. Caffrey, 2012. Structural insights into electron transfer in cca3-type cytochrome oxidase. Nature, 487(7408): 514-518.

Mahanama, R., A. Berenjian, P. Valtchev, A. Talbot, R. Biffin, H. Regtop, F. Dehghani and J.M. Kavanagh, 2011. Enhanced production of menaquinone 7 via solid substrate fermentation from *Bacillus subtilis*. International Journal of Food Engineering, 7(5).

Mahdinia, E., A. Demirci and A. Berenjian, 2018. Implementation of fed-batch strategies for vitamin k (menaquinone-7) production by *Bacillus subtilis* natto in biofilm reactors. Appl Microbiol Biotechnol, 102(21): 9147-9157.

Mielich-Suss, B. and D. Lopez, 2015. Molecular mechanisms involved in *Bacillus subtilis* biofilm formation.
Environ Microbiol, 17(3): 555-565.

Qin, Y., Y. He, Q. She, P. Larese-Casanova, P. Li and Y. Chai, 2019. Heterogeneity in respiratory electron transfer and adaptive iron utilization in a bacterial biofilm. Nat Commun, 10(1): 3702.

Que, Q. and J.D. Helmann, 2000. Manganese homeostasis in Bacillus subtilis is regulated by mntR, a bifunctional regulator related to the diphtheria toxin repressor family of proteins. Mol Microbiol, 35(6): 1454-1468.

Ranmadugala, D., A. Ebrahiminezhad, M. Manley-Harris, Y. Ghasemi and A. Berenjian, 2017. Impact of 3-aminopropyltriethoxysilane-coated iron oxide nanoparticles on menaquinone-7 production using B. subtilis. Nanomaterials (Basel), 7(11).

Ravishankar, B., Y.A. Dound, D.S. Mehta, B.K. Ashok, A. de Souza, M.-H. Pan, C.-T. Ho, V. Badmaev and A.D.B. Vaidya, 2015. Safety assessment of menaquinone-7 for use in human nutrition. Journal of Food and Drug Analysis, 23(1): 99-108.

Romero, D., C. Aguilar, R. Losick and R. Kolter, 2010. Amyloid fibers provide structural integrity to Bacillus subtilis biofilms. Proc Natl Acad Sci U S A, 107(5): 2230-2234.

Sato, T., L.J. Schurgers and K. Uenishi, 2012. Comparison of menaquinone-4 and menaquinone-7 bioavailability in healthy women. Nutrition journal, 11(1): 93.

Song, Y., J.M. Nikoloff and D. Zhang, 2015. Improving protein production on the level of regulation of both expression and secretion pathways in Bacillus subtilis. J Microbiol Biotech, 25(7): 963-977

Wang, H., H. Liu, L. Wang, G. Zhao, H. Tang, X. Sun, W. Ni, Q. Yang, P. Wang and Z. Zheng, 2019. Improvement of menaquinone-7 production by Bacillus subtilis natto in a novel residue-free medium by increasing the redox potential. Appl Microbiol Biotechnol, 103(18): 7519-7535.

Wilks, J.C., R.D. Kitko, S.H. Cleeton, G.E. Lee, C.S. Ugwu, B.D. Jones, S.S. BonDurant and J.L. Slonczewski,
2009. Acid and base stress and transcriptomic responses in *Bacillus subtilis*. Appl Environ Microbiol, 75(4): 981-990.

Wu, W.-J. and B.-Y. Ahn, 2011. Improved menaquinone (vitamin k2) production in cheonggukjang by optimization of the fermentation conditions. Food Science and Biotechnology, 20(6): 1585-1591.

Yang, S., Y. Cao, L. Sun, C. Li, X. Lin, Z. Cai, G. Zhang and H. Song, 2019. Modular pathway engineering of *Bacillus subtilis* to promote de novo biosynthesis of menaquinone-7. ACS Synth Biol, 8(1): 70-81.

Yang, Y., Y. Wu, Y. Hu, Y. Cao, C.L. Poh, B. Cao and H. Song, 2015. Engineering electrode-attached microbial consortia for high-performance xylose-fed microbial fuel cell. ACS Catalysis, 5(11): 6937-6945.

Yepes, A., J. Schneider, B. Mielich, G. Koch, J.C. Garcia-Betancur, K.S. Ramamurthi, H. Vlamakis and D. Lopez, 2012. The biofilm formation defect of a *Bacillus subtilis* flotillin-defective mutant involves the protease ftsh. Mol Microbiol, 86(2): 457-471.

Yu, J., L. Hederstedt and P.J. Piggot, 1995. The cytochrome bc complex (menaquinone:Cytochrome c reductase) in *Bacillus subtilis* has a nontraditional subunit organization. J Bacteriol, 177(23): 6751-6760.

Zhu, Y., L. Ye, Z. Chen, W. Hu, Y. Shi, J. Chen, C. Wang, Y. Li, W. Li and H. Yu, 2017. Synergic regulation of redox potential and oxygen uptake to enhance production of coenzyme q10 in rhodobacter sphaeroides. Enzyme and Microbial Technology, 101: 36-43.
Tables and Figures

Figure 1. Schematic diagram of cross-sectional structure of biofilm. Different types of cell subpopulations co-exist and exhibit different temporal and spatial distribution patterns. The genetic program associated with cell differentiation is shown in the box, and the genes associated with each differentiation process are located within a specific framework. The expression of yqxM-tasA operons is controlled by regulatory repressor SinI and anti-repressor protein SinR. The major proteinaceous component of matrix yuab and ftsh is controlled by DegS-DegU two component regulation system.

Figure 2. The relationship between biofilm formation and MK-7 titer. (A) The morphological changes of the biofilm when the key genes were knocked out, including single and double knockout of key genes. (B) The specific titer of MK-7 when biofilm formation genes were knocked out. The small image in the upper right corner is biomass. (C) Effect of double deletion of key genes in biofilm formation on specific titer of MK-7. All experiments were independently carried out at least three times and the results were expressed as mean ± standard deviation (SD).

Figure 3. The expression level of MK-7 synthesis related genes in static culture compared to shake culture. (A) Expression levels of genes involved in MK-7 synthesis, including glycolysis pathway (EMP), pentose phosphate pathway (PPP), shikimic acid (SHK) pathway, MK-7 pathway, 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway and tricarboxylic acid cycle.
(TCA). (B) Left: catalytic reaction of NADH reduction in cells, including L-lactate dehydrogenase (ldh), (R,R)-butanediol dehydrogenase (bdha), NADH dehydrogenase (ndh), aerobic glycerol-3-phosphate dehydrogenase (glpD) and succinate dehydrogenase flavoprotein (sdhABC). Right: expression level of NADH dehydrogenase in static culture, compared with shake culture, positive Numbers mean up-regulation, negative Numbers mean down-regulation. (C) Expression level of most dehydrogenases of cells in static culture, including various amino acid dehydrogenases. All experiments were independently carried out at least three times and the results were expressed as mean ± standard deviation (SD).

**Figure 4.** Transcriptomics analysis of differences between shake culture and static culture. (A) Volcanic maps show differentially expressed genes between groups. Red dots indicate up-regulated genes and blue dots indicate down-regulated genes. (B) Venn shows differentially expressed genes between each sample. (C) Gene Ontology (GO) functional analysis of differential genes. According to gene function, it can be divided into three categories: molecular function, cellular components and biological processes. The obvious differences in membrane part and catalytic activity. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of functions of differential genes. The most significant differences in cell metabolism category, include cofactors and vitamins metabolism, energy metabolism and amino acid metabolism. All experiments were independently carried out at least three times and the results were expressed as mean ± standard deviation (SD).

**Figure 5.** Overexpression of differentially expressed genes in *B. subtilis* 168 and BS20. (A)
Overexpression of differentially expressed genes in *B. subtilis* 168 and differences in biofilm morphology. (B) Effect of overexpression of differential genes on the titer of MK-7 in *B. subtilis* 168. The small image in the upper right corner is biomass. (C) Effect of combinatorial overexpression of differential genes on the titer of MK-7 in *B. subtilis* 168. (D) Overexpression of differentially expressed genes in the engineered strain BS20 and differences in biofilm morphology. (E) Effect of overexpression of differential genes on the titer of MK-7 in BS20. The small image in the upper right corner is biomass. (F) Effect of combinatorial overexpression of differential genes on the titer of MK-7 in BS20. All experiments were independently carried out at least three times and the results were expressed as mean ± standard deviation (SD). * and ** indicate p < 0.05 and p < 0.01, respectively.

**Figure 6.** Effect of electron transfer chain on the synthesis of MK-7. (A) Schematic diagram of electron transfer chain in *B. subtilis* 168. Electrons are extracted under action of NADH dehydrogenase, and MK-7 and cytochrome C act as electron transport carriers, and finally electrons are delivered to oxygen to form water. (B) Morphological differences in biofilms of *B. subtilis* 168 and BS20 overexpressed *qcrA-C* gene, respectively. (C) Changes in MK-7 production when overexpression of *qcrA-C* gene in *B. subtilis* 168 and BS20. QcrA*2* means that there are two copies in BS20. (D) Up: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of fermentation broth protein components, and there is a very distinct band at 46 KDa. Down: the translation level of OxdC increases with the fermentation time and SDS-PAGE verifies that *oxdC* was successfully knocked out in BS20. (E) Comparison of biofilm morphology of *B. subtilis* 168 and BS20 (*oxdC* deletion). (F) Changes in
MK-7 production and cell biomass of BS20 with oxdC deletion. (G) The relative expression levels of NADH dehydrogenase in the strain BS20 with oxdC deletion, and all the NADH dehydrogenases were up-regulated when oxdC was knocked out. (H) Changes in NADH and NAD content in the strain BS20 with oxdC deletion. All experiments were independently carried out at least three times and the results were expressed as mean ± standard deviation (SD). * and ** indicate p < 0.05 and p < 0.01, respectively.

**Figure 7.** The titer of MK-7 in 250 mL flask and 15-L bioreactor. (A) The titers of MK-7 synthesized by strains BS20, BS20-Q, BS20-T and BS20-QT. (B) The cell growth of BS20, BS20-Q, BS20-T and BS20-QT. (C) Effect of dissolved oxygen level (30% and 55%) on the synthesis of MK-7. (D) Effect of dissolved oxygen level (30% and 55%) on cell growth. We define that when the seed is not added, the dissolved oxygen level in the fermentation broth is 100%. All experiments were independently carried out at least three times and the results were expressed as mean ± standard deviation (SD). * and ** indicate p < 0.05 and p < 0.01, respectively.

**Table S2-S5 are additional Excel.**

Table S2. Compare with shake culture, the down-regulated genes were expressed in static culture. Related to Figure 4.

Table S3 Compare with shake culture, the up-regulated genes were expressed in static culture. Related to Figure 4.

Table S4 Gene Ontology (GO) functional annotation of differential genes. Related to Figure 4.
Table S5 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differential genes. Related to Figure 4.
Highlights

- Transcriptome analysis shows the relationship between biofilm and MK-7 synthesis.
- Electron transfer significantly affects the synthesis of MK-7.
- Oxalate decarboxylase OxdC plays a role in electron generation and MK-7 synthesis.