ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Efficient Biofilm-Based Fermentation Strategies by eDNA Formation for L-Proline Production with *Corynebacterium glutamicum*

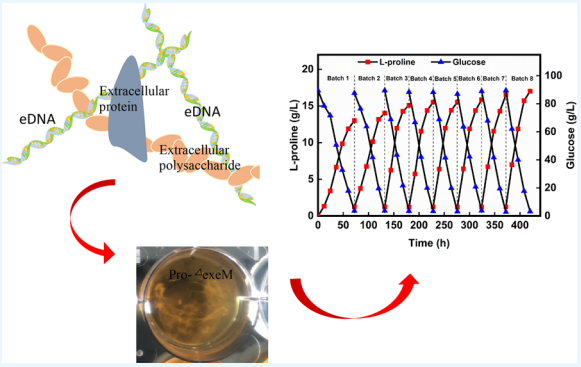
Peifang Ren, Tianpeng Chen, Na Liu, Wenjun Sun, Guang Hu, Ying Yu, Bin Yu, Pingkai Ouyang, Dong Liu,* and Yong Chen*

Cite This: *ACS Omega* 2020, 5, 33314−33322

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Biofilms could provide favorable conditions for the growth of cells during industrial fermentation. However, biofilm-immobilized fermentation has not yet been reported in *Corynebacterium glutamicum* (*C. glutamicum*), one of the main strains for amino acid production. This is mainly because *C. glutamicum* has a poor capability of adsorption onto materials or forming an extracellular polymeric substance (EPS). Here, an engineered strain, *C. glutamicum* Pro-Δ*exeM*, was created by removing the extracellular nuclease gene *exeM*, which effectively increased extracellular DNA (eDNA) in the EPS and cell adhesiveness onto carrier materials. In repeated-batch fermentation using the biofilm, L-proline production increased from 10.2 to 17.1 g/L. In summary, this research demonstrated that a synthetic *C. glutamicum* biofilm could be favorable for L-proline production, which could be extended to other industrial applications of *C. glutamicum*, and the strategy may also be applicable to the engineering of other strains.



1. INTRODUCTION

Biofilms, as microbial communities, are dynamic environments wherein cells propagate attached to organic or inorganic surfaces, as in dental plaque, food processing equipment, various water and ventilation pipes, medical equipment, etc.[1−3] Biofilms have some "social" attributes such as aggregate growth, adhesive medium, and quorum sensing.[4,5] Many strains on Earth can exist in the form of biofilms like *Clostridium acetobutylicum*, *Escherichia coli* (*E. coli*), *Bacillus subtilis*, and *Saccharomyces cerevisiae*.[6,7] The extracellular polymeric substance (EPS) matrix of biofilms mainly contains extracellular proteins, extracellular polysaccharides, and extracellular DNA (eDNA). These complex components determine the structure of biofilms and allow the bacteria in biofilms to adapt to a changing environment.[8,9] Recently, researchers have discovered that cells in the biofilm could grow steadily with high activity, providing favorable conditions for industrial fermentation.[10,11] Moreover, cells covered by biofilms could tolerate more hash conditions such as high osmotic pressure, oxygen limitations, and high cell density during fermentation.[12] Subsequently, biofilm-immobilized fermentation was proposed and applied in the fermentation industry by improving strain adsorption capacity.

During biofilm-immobilized fermentation, the biofilm cells can withstand high-speed agitation.[13] Furthermore, the biofilm cells that attach to the carrier surface could be renewed when fermentation broth is replaced with a fresh medium.[14] Due to high cell activity and the repetitive usage of cells in the biofilm-immobilization fermentation, seed culture can be eliminated, the lag phase can be reduced, and the production cycle could be shortened substantially.[15]

In biofilms, eDNA plays an important role in the connection among the carriers, cells, extracellular polysaccharides, and proteins.[16,17] eDNA was shown to have a great influence on the biofilm structure of *Streptococcus mutans* and *Staphylococcus aureus*.[18,19] Sumei Liao et al.[20] found that eDNA could be woven with extracellular polysaccharides to form an inseparable flocculent structure in *Streptococcus mutans* biofilms. In addition, eDNA could maintain the extracellular biofilm matrix[21,22] and acted as a "kite line" that restricted the outward release of the bacteria at the top of the biofilm structure. eDNA participates in the entire life cycle of biofilms from the initial stage of formation to the dissociation.[23] So, eDNA was not only the key to biofilm formation but also the main component maintaining biofilm structure.[24] Therefore,

Received: October 19, 2020
Accepted: December 3, 2020
Published: December 15, 2020

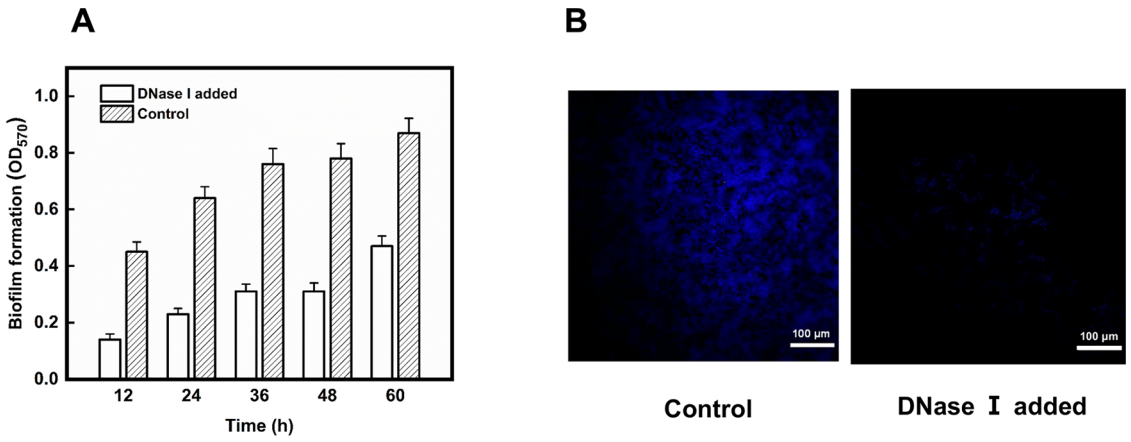


Figure 1. Effect of addition of DNase I on biofilm formation in the original strain. (A) Effect of DNase I on the biofilm amount during the fermentation process. DNase I (10 μL) (5 U) and 20 μL of seed culture were added to each well of a 96-well plate with 180 μL of the fermentation medium. Another group of samples with inactivated DNase I was used as a negative control. The biofilm amount was detected at the 12, 24, 36, 48, and 60 h time points. (B) CLSM images of biofilms, which were cultivated for 72 h at 30 °C with DNase I.

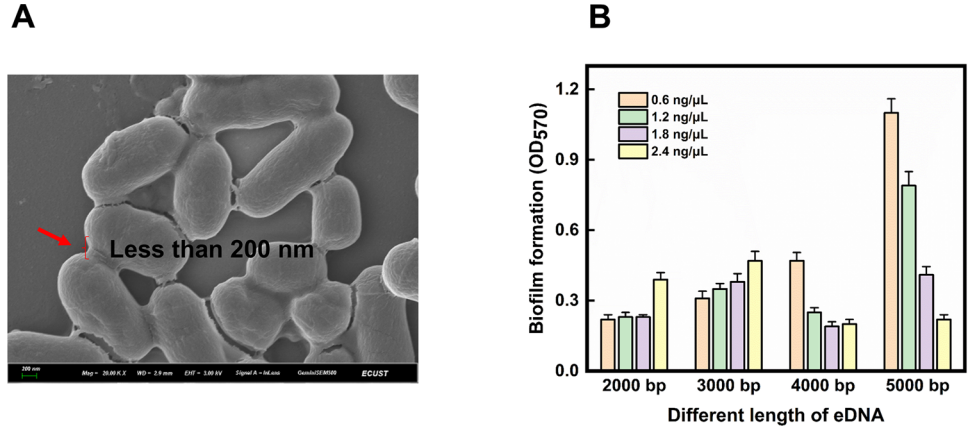


Figure 2. EPS matrix of *C. glutamicum* ATCC 13032-ProB cells and quantitative analysis of the effect of DNA addition on biofilm formation in the original strain. (A) Distance of *C. glutamicum* ATCC 13032-ProB cells in the biofilm, as indicated by the red signs. The biofilm was formed on a cell slide in a 6-well plate after 72 h of cultivation at 30 °C. (B) Effects of added DNA fragments on biofilm formation. DNA fragments with different lengths (2000, 3000, 4000, or 5000 bp) and different final concentrations (0.6, 1.2, 1.8, and 2.4 μg/mL) were added at the beginning of cultivation. Experiments were carried out in 96-well plates, and biofilm was quantified after 72 h of incubation at 30 °C.

an increase in eDNA accumulation would be beneficial to biofilm formation. Extracellular nuclease is one of the factors responsible for the degradation of eDNA.[20] Researchers have shown that the removal of nuclease genes *exeM* and *exeS* promoted the accumulation of eDNA and biofilm formation in *Shewanella oneidensis* (*S. oneidensis*) MR-1.[25] On the other hand, many bacteria have a type IV secretion system (T4SS) that could secret DNA or DNA-protein complexes.[26,27] Within the T4SS, the *VirB11* family proteins were the most important for the DNA and protein to go through.[28−30] Although there was no research on *exeM* or *VirB11* in *Corynebacterium glutamicum* (*C. glutamicum*), in this study, we identified two genes highly homologous to *exeM* and *VirB11* in *C. glutamicum* ATCC 13032-ProB, which produces L-proline.[31] Subsequently, we engineered the *C. glutamicum* ATCC 13032-ProB strain to enhance biofilm formation by removing *exeM* and overexpressing *VirB11*. Overall, this study represents a successful case for the development of biofilm-based fermentation under aerobic (nonstatic) industrial conditions for efficient biochemical production.

2. RESULTS

2.1. Effect of Extracellular DNA on Biofilms. DNA fragments could be degraded by DNase I effectively without influencing the cell growth.[17] Thus, 10 μL of DNase I (5 U) was added to each well in order to reduce the DNA content within *C. glutamicum* ATCC 13032-ProB biofilms at various intervals. As expected, samples with inactivated DNase I showed a better biofilm formation ability, which indicated that the eDNA played an important role throughout the whole process of biofilm formation (Figure 1A). In addition, some researches verified the effects of DNase I on the biofilm matrix, which had been formed completely.[17] The cells in biofilms were stained with DAPI and detected by a CLSM (Figure 1B). The morphological structure of the biofilm with inactivated DNase I was tighter and more uniform (Figure 1B, left) compared with that of the biofilm that had added active DNase I (Figure 1B, right).

Extracellular secretions were observed, and the distance of two adjacent cells in the biofilm was less than 200 nm (Figure 2A). So, a length of 2000 bp DNA fragment (680 nm) would be sufficient to connect adjacent cells. Results showed that the

https://dx.doi.org/10.1021/acsomega.0c05095
ACS Omega 2020, 5, 33314−33322

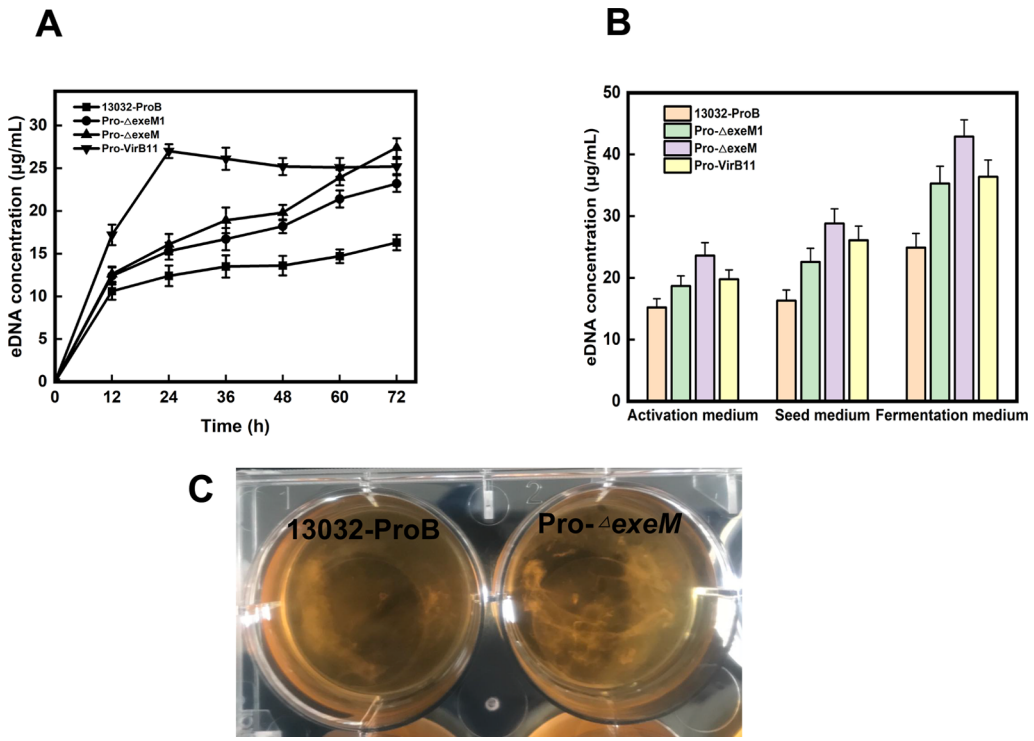


Figure 3. Comparison of eDNA accumulation and biofilm formation between the recombinant strains and the original strain. The incubation time and conditions were the same as in Figure 2A. *C. glutamicum* ATCC 13032-ProB was abbreviated to 13032-ProB in the legends. (A) Differences in eDNA formation between the four strains during the cultivation process. (B) Biofilm formation ability of the four strains under different media. (C) Visible biofilm observed for the Pro-Δ*exeM* strain but not for the original strain (13032-ProB).

biofilm formation with different concentrations (0.6−2.4 ng/μL) and lengths (2000−5000 bp) of added DNA was all stronger than that without the DNA addition (OD_{570} = 0.12). In particular, 0.6 ng/μL 5000 bp DNA gave the most apparent biofilm formation (Figure 2B).

2.2. Extracellular DNA Content of Engineered Strains. PCR and sequencing results confirmed that the three recombinant strains in which the *VirB11* gene was overexpressed (Pro-*VirB11*) or the *exeM* gene was knocked out (Pro-Δ*exeM*1, Pro-Δ*exeM*) were constructed successfully. The eDNA concentration of the original and three recombinant strains increased rapidly in the first 12 h and then increased steadily throughout the fermentation stage. At the end of the batch fermentation, the eDNA concentration of the original bacteria, Pro-Δ*exeM*1, Pro-Δ*exeM*, and Pro-*VirB11* were 16.3, 23.2, 27.4, and 25.2 μg/mL, respectively. Pro-Δ*exeM* had the highest amount of extracellular DNA that was 68.1% higher than the original strain (Figure 3A).

The 96-well plate experiment showed different biofilm formation abilities of the strains, as was detected by the crystal violet semi-quantitative method using the fermentation medium. The optical density from the crystal violet staining (which was an indicator of biofilm quantity) for Pro-Δ*exeM* was increased by 75% compared with the original strain (2.17 vs 1.24), while Pro-Δ*exeM*1 was increased by 41.9% (1.76 vs 1.24). Similar results were found in Pro-*VirB11*, which exhibited an increase of 41.1% (1.75 vs 1.24). The activation medium (LBG) and the seed medium were tried as well, and similar results were observed (Figure 3B).

The biofilm formed by the Pro-Δ*exeM* strain, which had the highest eDNA content, could be visibly observed (Figure 3C) after being cultured for 72 h in a fermentation medium in a 6-well plate, whereas the biofilm formation by the original strain could not be observed under such methods.

2.3. Biofilm Observation. Weak fluorescence was observed in the original strain by a DAPI fluorescent dye (Figure 4A), while relatively strong fluorescence was found in the recombinant strains Pro-Δ*exeM*1 (Figure 4B), Pro-Δ*exeM* (Figure 4C), and Pro-*VirB11* (Figure 4D). This indicated that the biofilm in the original strain was scarce and scattered, whereas the biofilm of the recombinant strains was thick and abundant (Figure 4).

DDAO staining and subsequent CLSM indicated that extracellular DNA surrounded the recombinant cells in the biofilm. The red staining of eDNA by DDAO in Pro-Δ*exeM* (Figure 5A-2) and Pro-*VirB11* (Figure 5A-3) was significantly stronger and wider than the original strain (Figure 5A-1). An overlay of bright fields and dark fields showed that more cells were colocalized with clumps of eDNA (Figure 5A). A 3D recontruction of the eDNA staining of Pro-Δ*exeM* revealed that a large amount eDNAs formed an extensive three-dimensional complex structure filled with holes and extrusions (Figure 5B).

2.4. Biofilm-Based Fermentation for Enhanced L-Proline Production. The *exeM* involved in eDNA accumulation did not affect the metabolic capacity of strains. Simultaneously, the Pro-Δ*exeM* strain showed a greater ability in its biofilm formation. Thus, the Pro-Δ*exeM* strain was chosen for immobilized repeated-batch fermentation.

L-Proline production in the first four batches had improved gradually in Pro-Δ*exeM* (Figure 6A). After the fourth batch, L-proline production was maintained at an average of 16.2 g/L, and L-proline productivity was kept at around 0.34 g/L/h, which was much higher compared with that of the original

https://dx.doi.org/10.1021/acsomega.0c05095
ACS Omega 2020, 5, 33314−33322

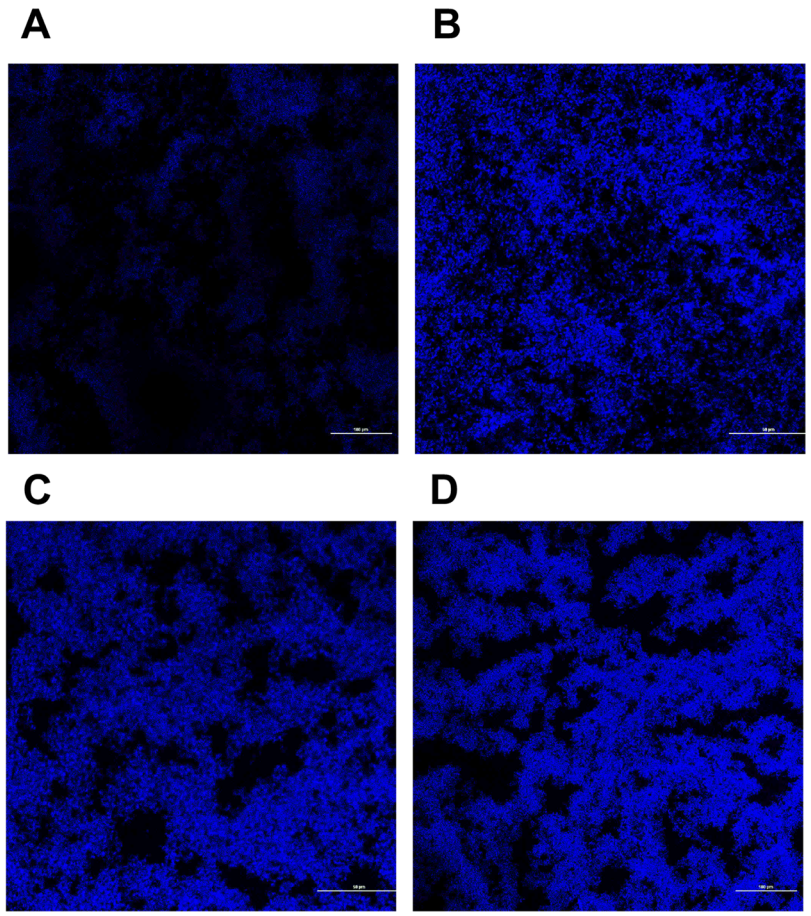


Figure 4. Confocal laser scanning microscopy images of the biofilm formed by different strains. The biofilm was stained with a DAPI fluorescent dye. The incubation time and conditions were the same as Figure 2A. (A) *C. glutamicum* ATCC 13032-ProB, (B) Pro-Δ*exeM*1, (C) Pro-Δ*exeM*, and (D) Pro-*VirB11*.

strain (Figure 6B) (0.34 g/L/h vs 0.14 g/L/h). Through a fermentation process of 420 h, L-proline production was increased by 66% in Pro-Δ*exeM* compared with that of the original strain (17.1 g/L vs 10.3 g/L). Furthermore, the fermentation period was shortened from 72 to 48 h. Therefore, the immobilized repeated-batch fermentation taking advantage of the biofilm formation in the *exeM*-knockout strain could enhance the L-proline concentration and productivity.

3. DISCUSSION

C. glutamicum ATCC 13032-ProB was used to establish a biofilm-based fermentation for production of L-proline. This original strain formed less biofilms, which limited its industrial application.

3.1. Extracellular DNA for Enhanced Biofilm Formation. The original strains formed less biofilms because the extracellular DNase I hydrolyzed the eDNA during the biofilm formation process or because the biofilm could not form due to insufficient eDNA at the initial stage. In the culture medium, different lengths and concentrations of added DNA were all demonstrated to promote the formation of biofilms. This suggested that addition of exogenous DNA would affect biofilms positively. In particular, the results showed that a low dose (0.6 ng/μL) of longer DNA fragments (5000 bp) could be more beneficial for biofilm formation.

3.2. Molecular Modification of Genes. We identified the gene homologs of *exeM* in *S. oneidensis* MR-1 by protein blast from NCBI and searched the *VirB11* gene in *C. glutamicum* ATCC 13032 from NCBI. The knockout of *exeM* and overexpression of *VirB11* resulted in significant improvement in the formation of biofilms. It was uncertain that the knockout Pro-Δ*exeM*1 strain has removed one *exeM* gene or two *exeM* genes. However, we removed all *exeM* genes in the Pro-Δ*exeM* strain.

Furthermore, the Pro-Δ*exeM* strain produced the most eDNA and biofilms, suggesting that reducing the extracellular nucleases might be more effective than trying to promote the secretion of eDNA through *VirB11*.

3.3. Biofilm-Based Immobilized Fermentation. The biofilm-based immobilized fermentation strategy was widely applied in industrial production.[32] The Pro-Δ*exeM* strain with a better biofilm formation ability was selected for the immobilized fermentation of L-proline, and it achieved greater production levels compared to the other strains. The Pro-Δ*exeM* would be of great value for the immobilized fermentation of L-proline, and the addition of a biofilm carrier was also indispensable. The carrier could fix bacteria on its surface and support biofilm development, but it had to be suitable for oxygen and mass transfer during the cell growth process.[33] The bacteria cells in the liquid fermentation broth were dramatically decreased, which was beneficial for product separation. Compared with free-cell fermentation, the carrier with attached biofilms served as an immobilized biocatalyst that could be reused again and again, with a cell renewal ability.

https://dx.doi.org/10.1021/acsomega.0c05095
ACS Omega 2020, 5, 33314−33322

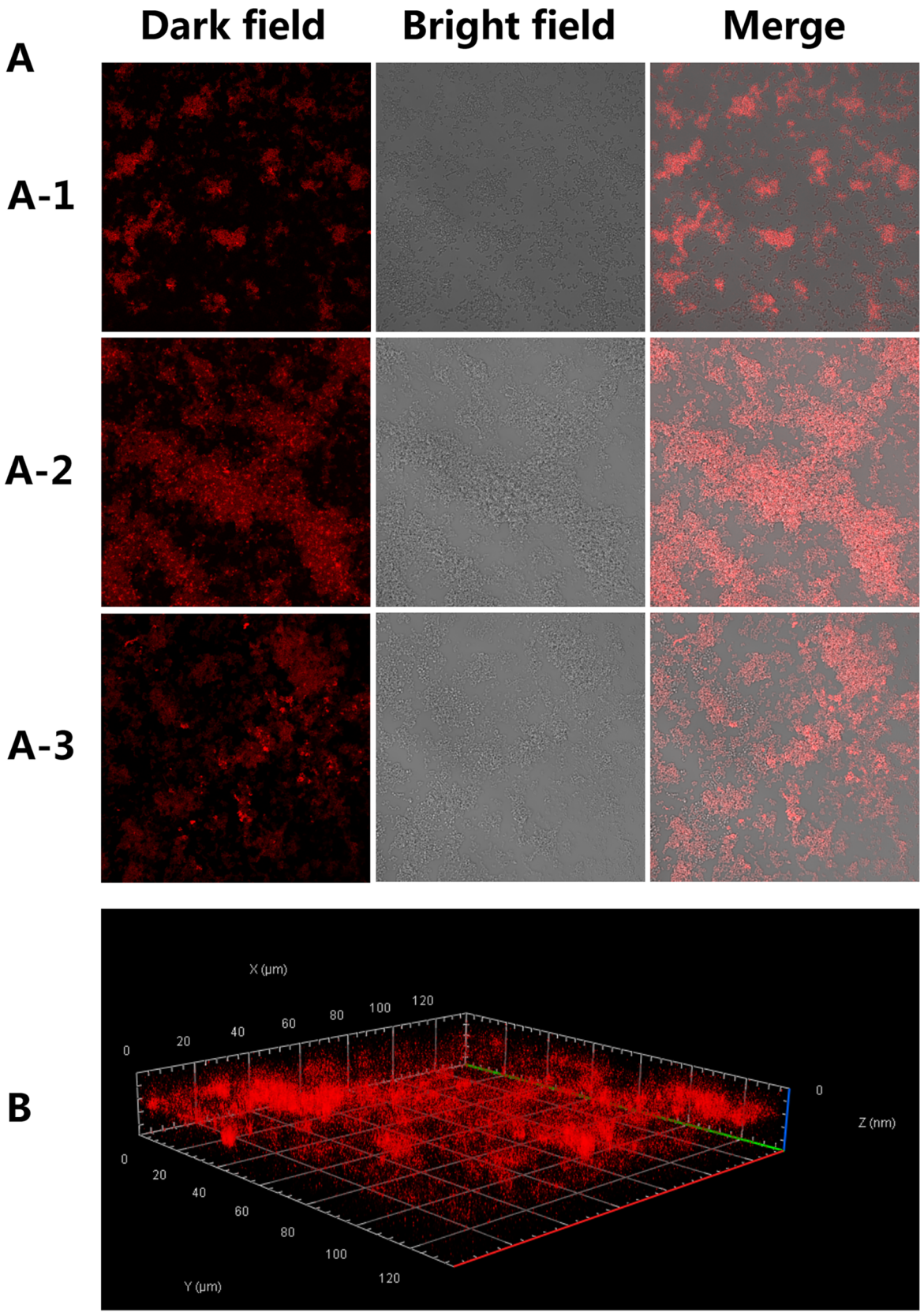


Figure 5. Confocal laser scanning microscopy images of three different strains stained with a DDAO fluorescent dye. The incubation time and conditions were the same as Figure 2A. (A) CLSM images of the eDNA in different strains at different fields. (A-1) *C. glutamicum* ATCC 13032-ProB, (A-2) Pro-Δ*exeM*, and (A-3) Pro-*VirB11*. (B) Three-dimensional image of eDNA in Pro-Δ*exeM* stained with a DDAO fluorescent dye.

This eliminated the need for seed culture. However, biofilm reactors will be complicated by the biofilm carrier. Modification and screening of carriers should also be carried out in the future. This study would also provide a reference for

https://dx.doi.org/10.1021/acsomega.0c05095
ACS Omega 2020, 5, 33314−33322

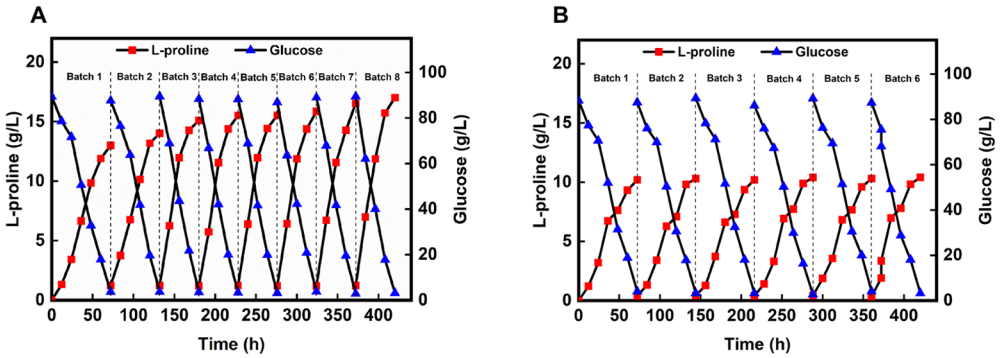


Figure 6. Biofilm-based immobilized fermentation. Polyurethane foam at 30 g/L was added as a biofilm carrier. At the end of each batch, the fermented broth was removed, and the biofilm carrier was left for repeated use in the next batch that was initiated by adding a fresh culture medium. (A) L-proline production by Pro-Δ*exeM*. (B) L-proline production by *C. glutamicum* ATCC 13032-ProB.

developing more biochemical-producing processes based on *C. glutamicum* biofilms and applying eDNA to other biofilm-forming bacteria.

4. MATERIALS AND METHODS

4.1. Genes, Strains, and Plasmids. The research strain *C. glutamicum* ATCC 13032-ProB is a derivative from *C. glutamicum* ATCC 13032. The genes, strains, and plasmids used in this work are listed in Table 1, and the primers are in Table 2.

4.2. Gene Source. The *exeM* gene in *S. oneidensis* MR-1 was used to blast proteins in *C. glutamicum* ATCC 13032 by NCBI. An *ExeM/NucH* family extracellular endonuclease (CGL-RS12940) was found and was named *exeM* in this study. On the other hand, a gene (Cg10301) predicted to encode a *VirB11* family ATPase, involved in pili and flagella biosynthesis, was named *VirB11* in this study.

Table 1. Bacterial Strains, Plasmids and Genes Used in This Study

strains	relevant characteristics	source
C. glutamicum ATCC 13032-ProB[a]	original strain, L-proline producer	Prof. Sheng Yang
E. coli DH5α-pXMJ19	Cm resistance	stored in our lab
E. coli DH5α-pk18mobsacB	Kan resistance	stored in our lab
Pro-Δ*exeM*1	*C. glutamicum* ATCC 13032-ProB with deleted part *exeM*	this study
Pro-Δ*exeM*	*C. glutamicum* ATCC 13032-ProB with deleted *exeM*	this study
Pro-*VirB11*	*C. glutamicum* ATCC 13032-ProB harboring plasmid pXMJ19-*VirB11*	this study
Plasmids		
pk18mobsacB	Kan resistance	this study
pXMJ19	Cm resistance	this study
pk18mobsacB-*exeM*	pk18mobsacB with Δ*exeM* from *C. glutamicum* ATCC 13032-ProB	this study
pXMJ19-*VirB11*	pXMJ19 with *VirB11* from *C. glutamicum* ATCC 13032-ProB	this study
Genes		
exeM	CGL-RS12940, *ExeM/NucH* family extracellular endonuclease	NCBI
VirB11	Cg10301, predicted ATPases involved in pili and flagella biosynthesis, *VirB11* family	NCBI

[a]A gift from Prof. Sheng Yang (Institute of Plant Physiology & Ecology, CAS, Shanghai, China).

4.3. Strain Engineering. One or two copies of the *exeM* gene in the parental strain were removed by the long flanking homology region-PCR (LFH-PCR) method[34] to obtain a strain named Pro-Δ*exeM*1. The PCR primers were designed by using the National Center for Biotechnology Information (NCBI) gene sequence database and SnapGene design. Briefly, a PCR-generated Chloramphenicol resistance marker was used as a knock-in DNA fragment. The Chloramphenicol resistance marker consisted of a Chloramphenicol resistance sequence in homologous regions (around 1500 bp) flanking the target locus.

Complete removal of *exeM* was achieved by using a pk18mobsacB plasmid. The resulting strain was named Pro-Δ*exeM*.[35] Briefly, a PCR-generated Kanamycin resistance marker was used as a knock-in DNA fragment. The Kanamycin resistance marker consisted of a Kanamycin resistance sequence in plasmid pk18mobsacB and homologous regions (around 1500 bp) flanking the target locus. The knock-in component, which was named pk18mobsacB-*exeM*, was transformed into strain *C. glutamicum* ATCC 13032-ProB using a Bio-Rad electroporation system set at 1.8 kV and 25 mF with a 200 Ohm pulse controller.

The *VirB11* gene was amplified from the genome DNA of *C. glutamicum* ATCC 13032-ProB and ligated to the over-expression plasmid pXMJ19 (*BamH* I) to obtain the plasmid pXMJ19-*VirB11*. The *VirB11* gene and plasmid pXMJ19 (with restriction enzyme *BamH* I) were ligated by using the ClonExpress II One Step Cloning Kit C112-01 (Vazyme, Nanjing, China), resulting in a plasmid pXMJ19-*VirB11*. The final engineered strain was named Pro-*VirB11* with Chloramphenicol resistance for screening.

4.4. Media and Growth Conditions. *C. glutamicum* ATCC 13032-ProB, Pro-Δ*exeM*1, Pro-Δ*exeM*, and Pro-*VirB11* were cultured in an LBG medium containing 5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, and 10 g/L glucose. The agar plates were prepared by adding 1.5% (w/v) agar into LBG media. The seed medium contained 25 g/L glucose, 17.5 g/L corn steep liquor, 5 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L KH_2PO_4, and 2 g/L urea. The fermentation medium contained 100 g/L glucose, 20 g/L corn steep liquor, 30 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 1.2 g/L KH_2PO_4, 2 g/L urea, and 15 g/L $CaCO_3$, and 2.4 g/L urea was added to the medium every 12 h. Acetic acid was added to the medium to adjust its initial pH level to 7.2. Kanamycin (50 mg/mL),

https://dx.doi.org/10.1021/acsomega.0c05095
ACS Omega 2020, 5, 33314−33322

Table 2. Main Primers Used in This Study

primers	relevant characteristics
*exeM*1-up-F	TCACTTCGCGACCTCAACTC
*exeM*1-up-R	agtggcagggcggggcgtaaACAGACAATTTGTTGCTGGT
*exeM*1-down-F	ccagtgatttttttctccatgcatTGGCAGCCTCGACCAC
*exeM*1-down-R	CAGACCTGCGAGCCGACGGC
Cm-*exeM*1-F	ACCAGCAACAAATTGTCTGTttacgccccgccctgccact
Cm-*exeM*1-R	GTGGTCGAGGCTGCCAATGCatggagaaaaaaatcactggatataccaccgttgat
exeM-up-F	aattcgagctcggtacccggggatccTCACTTCGCGACCTCAACTCCC
exeM-up-R	ACAGACAATTTGTTGCTGGTCTCAGG
exeM-down-F	CCTGAGACCAGCAACAAATTGTCTGTGCATTGGCAGCCTCGACCACG
exeM-down-R	gcctgcaggtcgactctagaggatccCAGACCTGCGAGCCGACGG
VirB11-F	gcctgcaggtcgactctagaggatccATGACTGACATTGATCTGGTGGTGGAA
VirB11-R	aattcgagctcggtacccggggatccCTAGGGCATAAACCATGCCTCTTCG
Y-*exeM*-F	TCACTTCGCGACCTCAACTC
Y-*exeM*-R	CAGACCTGCGAGCCGACGGC
Y-*VirB11*-F	aattaagcttgcatgcctgcaggt
Y-*VirB11*-R	atcggcgctacggcgtttca

Chloramphenicol (50 mg/mL), sucrose (10 g/L), isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) or L-arabinose (100 mM) was added as required. The fermentation culture was grown at 30 °C with an agitation speed of 220 rpm.

For the free-cell fermentation, the flasks were inoculated with 6 to 9% (v/v) seed culture and then shaken for 72 h at 220 rpm in 30 °C. The culture was sampled every 12 h to monitor the glucose and L-proline.

The same conditions were used for immobilized repeated-batch fermentation with 30 g/L polyurethane foam as a biofilm carrier. At the end of the first batch, the fermented broth was removed from the flask, and the carrier that was covered by biofilms was left for the second batch. After adding a fresh culture medium, the second batch was initiated under the same conditions. The subsequent batches were operated in the same way as above.

4.5. Carrier Preparation. A novel porous polyurethane foam was prepared in the laboratory. The carrier had a density of 0.63 g/cm^3 with a pore diameter of 0.2 to 0.4 mm sheared to a size of 5 mm × 5 mm × 5 mm. This carrier was pretreated using the previously reported method.[36] The carrier was rinsed in 1 M NaOH and then 1 M HCl before being washed with sterile water until the pH value reached 7.0. The carrier was sterilized at 121 °C for 15 min before use.

4.6. Analytical Methods. L-Proline was measured by HPLC (Agilent 1260 series; Hewlett-Packard, Palo Alto, CA, USA) with a UV detector, using a Sepax AAA ion exclusion column (250 × 4.6 mm; Bio-Rad Laboratories, Hercules, CA, USA), with sodium acetate and 80% acetonitrile as the mobile phase (0.6 mL/min) at 39 °C. In this study, we used triethylamine acetonitrile, phenyl isothiocyanate acetonitrile, and *n*-hexane to make L-proline become weak-polar derivatives that could be detected by HPLC. Glucose was measured by a refractive index detector by using an Aminex HPX-87H column (300 × 7.8 mm), with 5 mM H_2SO_4 as the mobile phase (1 mL/min) at 55°C.[37]

After the cell culture matured, the medium was discarded, and the 96-well plate was rinsed twice with PBS (1%) and prepared for washing the free cells. Methanol (200 μL) was used for fixing the biofilm for 10 min. Then, methanol was poured out, and the 96-well plate was dried at room temperature. The biofilm was stained with 200 μL of 2% crystal violet for 10 min at room temperature. After that, all wells were rinsed gently with PBS three times and patted lightly on absorbent paper towels, and then, the plate was left to dry at room temperature or inside an incubator at 30 °C. For semi-quantitative purposes, 200 μL of 30% glacial acetic acid was added, and the wells were incubated by agitating gently for 30 min. The absorbance of crystal violet was an indicator of biofilm quantity, and this was measured at 570 nm by using a multiscan spectrum (SpectraMax iD5; Molecular Devices, USA).[38]

eDNA was extracted by the Gram-positive bacteria genome extraction kit (TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver.3.0). To quantify the eDNA amount, denatured fish sperm DNA solution (Sangon Biotech B548210, 5 mg/mL) was combined with a Hoechst 33258 fluorescent dye and used to draw a standard curve.[39,40] The maximum excitation wavelength and maximum emission wavelength were determined in the microplate reader. The standard curve equation was $y = 2411.3x + 3059.8$ ($R^2 = 0.9915$), and the linear range was 0.1−1.0 μg/mL. Samples that contained 1 mL of extracted eDNA were diluted 50 times with an equal volume of TNE buffer (pH 7.4) and a Hoechst 33258 fluorescent dye. These were mixed evenly and shielded from light at room temperature for 5 min. Each sample (200 μL) was immediately added to a black 96-well plate, and then, the DNA concentration was detected by the microplate reader.

A confocal laser scanning microscope (CLSM) (Leica TCS SP5II, Wetzlar, Germany) was used to visualize the distribution of biofilms and the content of eDNA. The cell slide was gently removed from the fermentation broth in the 6-well plate. PBS (1%) was used to wash the slides twice; then, 2.5% glutaraldehyde was used to fix the biofilm for 30 min; then, PBS (1%) was used to wash the biofilm three times. 4′,6-Diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA)[41] and 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) (AAT Bioquest, CA, USA)[42] fluorescent dyes (0.2 μg/mL) were added. They were left to settle and stain the nuclei and eDNA for 20 min; then, PBS (1%) was used to wash them three times. An anti-fluorescence quencher (50 μL) was applied on the cell slide after the cell slide was dried at room temperature in the dark. Finally, the cell slide glass was sealed with nail polish and observed under the CLSM immediately.

https://dx.doi.org/10.1021/acsomega.0c05095
ACS Omega 2020, 5, 33314−33322

Two fluorescent dyes were used to visualize the biofilm morphology. A DAPI fluorescent dye was used to stain the nucleus of the strains that were observed. A DDAO fluorescent dye was used to stain the eDNA of the three strains. DAPI was able to penetrate the cell membranes and bound to double-stranded DNA with strong bluish fluorescence. DDAO was unable to penetrate the cell membranes but could bind to extracellular double-stranded DNA with strong red fluorescence.

A field emission scanning electron microscope (FESEM) (SEM JSM-6360LV, Jeol Ltd., Japan) was used to visualize the biofilm morphology. The cell slide with attached biofilms was taken out from the 6-well plate and washed three times with PBS (1%). Glutaraldehyde (2.5%) was used to fix the biofilm at 4 °C for 12 h, and then, PBS (1%) was used to wash the cell slide. The cell slide was placed in the refrigerator at −80 °C overnight; then, it was dehydrated by a vacuum freeze-drying device (Labconco Corporation, Fort Scott, Kansas, USA) and coated with gold−palladium before it was placed in an FESEM for analysis.

AUTHOR INFORMATION

Corresponding Authors

Dong Liu − *State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering and National Engineering Research Center for Biotechnology, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, P.R. China*; orcid.org/0000-0002-3009-7590; Email: liudong@njtech.edu.cn

Yong Chen − *State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering and National Engineering Research Center for Biotechnology, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, P.R. China*; orcid.org/0000-0001-6913-6510; Email: chenyong1982@njtech.edu.cn

Authors

Peifang Ren − *State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, P.R. China*

Tianpeng Chen − *State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, P.R. China*

Na Liu − *State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, P.R. China*

Wenjun Sun − *State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, P.R. China*

Guang Hu − *Nanjing Iaso Biotherapeutics Co., Ltd., Nanjing 210000, P.R. China*

Ying Yu − *State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, P.R. China*

Bin Yu − *State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, P.R. China*

Pingkai Ouyang − *State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering and National Engineering Research Center for Biotechnology, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, P.R. China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c05095

Author Contributions

P.R. conceived and designed the experiments, performed the laboratory work, and analyzed the data. T.C. constructed the plasmids and strains, participated in the fermentation experiments, and drafted the paper. N.L. performed the shooting of the electron microscope. W.S. revised the graphs. G.H. revised the manuscript critically. Y.Y. analyzed the data. B.Y. supplied the carriers. P.O. revised the manuscript. Y.C. and D.L. contributed to experimental design and data interpretation. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Prof. Sheng Yang for providing strain *C. glutamicum* ATCC 13032-ProB. We also gratefully acknowledge the support of the National Key Research and Development Program of China (grant nos. 2018YFA0902200 and 2018YFB1501705), the National Natural Science Foundation of China (grant no. 21636003), the Outstanding Youth Foundation of Jiangsu (grant no. SBK2017010373), the Program for Changjiang Scholars and Innovative Research Team in University (IRT_14R28), the Jiangsu National Synergetic Innovation Center for Advanced Materials (SICAM), the Technology Support Program of Jiangsu (grant no. BE2014715), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

REFERENCES

(1) Douglas, L. J. Candida biofilms and their role in infection. *Trends Microbiol.* **2003**, *11*, 30−36.

(2) O'toole, G.; Kaplan, H. B.; Kolter, R. Biofilm formation as microbial development. *Annu. Rev. Microbiol.* **2000**, *54*, 49−79.

(3) Mah, T.-F. C.; O'Toole, G. A. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* **2001**, *9*, 34−39.

(4) Connell, J. L.; Whiteley, M.; Shear, J. B. Sociomicrobiology in engineered landscapes. *Nat. Chem. Biol.* **2012**, *8*, 10−13.

(5) Renner, L. D.; Weibel, D. B. Physicochemical regulation of biofilm formation. *MRS Bull.* **2011**, *36*, 347−355.

(6) Donlan, R. M.; Costerton, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **2002**, *15*, 167−193.

(7) Costerton, J. W.; Stewart, P. S.; Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* **1999**, *284*, 1318−1322.

(8) Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2004**, *2*, 95.

(9) Shi, X.; Chen, Y.; Ren, H.; Liu, D.; Zhao, T.; Zhao, N.; Ying, H. Economically enhanced succinic acid fermentation from cassava bagasse hydrolysate using *Corynebacterium glutamicum* immobilized in porous polyurethane filler. *Bioresour. Technol.* **2014**, *174*, 190−197.

(10) Mielich-Süss, B.; Lopez, D. Molecular mechanisms involved in *Bacillus subtilis* biofilm formation. *Environ. Microbiol.* **2015**, *17*, 555−565.
(11) Cairns, L. S.; Hobley, L.; Stanley-Wall, N. R. Biofilm formation by *Bacillus subtilis*: new insights into regulatory strategies and assembly mechanisms. *Mol. Microbiol.* **2014**, *93*, 587−598.
(12) Yamaguchi, T.; Ishida, M.; Suzuki, T. An immobilized cell system in polyurethane foam for the lipophilic micro-alga *Prototheca zopfii*. *Process Biochem.* **1999**, *34*, 167−171.
(13) De, O. I.; Romero, L. E.; Cantero, D. Optimization of immobilization conditions for vinegar production. Siran, wood chips and polyurethane foam as carriers for Acetobacter aceti. *Process Biochem.* **2004**, *39*, 547−555.
(14) Huang, Y. L.; Wu, Z.; Zhang, L.; Cheung, C. M.; Yang, S.-T. Production of carboxylic acids from hydrolyzed corn meal by immobilized cell fermentation in a fibrous-bed bioreactor. *Bioresour. Technol.* **2002**, *82*, 51−59.
(15) Kim, K. H.; Choi, I. S.; Kim, H. M.; Wi, S. G.; Bae, H. J. Bioethanol production from the nutrient stress-induced microalga *Chlorella vulgaris* by enzymatic hydrolysis and immobilized yeast fermentation. *Bioresour. Technol.* **2014**, *153*, 47−54.
(16) Allesen-Holm, M.; Barken, K. B.; Yang, L.; Klausen, M.; Webb, J. S.; Kjelleberg, S.; Molin, S.; Givskov, M.; Tolker-Nielsen, T. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol. Microbiol.* **2006**, *59*, 1114−1128.
(17) Dengler, V.; Foulston, L.; Defrancesco, A. S.; Losick, R. An electrostatic net model for the role of extracellular DNA in biofilm formation by *Staphylococcus aureus*. *J. Bacteriol.* **2015**, *197*, 3779−3787.
(18) Fischer, A.; Kambara, K.; Meyer, H.; Stenz, L.; Bonetti, E.-J.; Girard, M.; Lalk, M.; Francois, P.; Schrenzel, J. *GdpS* contributes to *Staphylococcus aureus* biofilm formation by regulation of eDNA release. *Int. J. Med. Microbiol.* **2014**, *304*, 284−299.
(19) Das, T.; Sharma, P. K.; Krom, B. P.; van der Mei, H. C.; Busscher, H. J. Role of eDNA on the adhesion forces between *Streptococcus mutans* and substratum surfaces: influence of ionic strength and substratum hydrophobicity. *Langmuir* **2011**, *27*, 10113−10118.
(20) Liao, S.; Klein, M. I.; Heim, K. P.; Fan, Y.; Bitoun, J. P.; Ahn, S.-J.; Burne, R. A.; Koo, H.; Brady, L. J.; Wen, Z. T. *Streptococcus mutans* extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery. *J. Bacteriol.* **2014**, *196*, 2355−2366.
(21) Huseby, M. J.; Kruse, A. C.; Digre, J.; Kohler, P. L.; Vocke, J. A.; Mann, E. E.; Bayles, K. W.; Bohach, G. A.; Schlievert, P. M.; Ohlendorf, D. H.; Earhart, C. A. Beta toxin catalyzes formation of nucleoprotein matrix in *Staphylococcal* biofilms. *Proc. Natl. Acad. Sci.* **2010**, *107*, 14407−14412.
(22) Rohde, H.; Burandt, E. C.; Siemssen, N.; Frommelt, L.; Burdelski, C.; Wurster, S.; Scherpe, S.; Davies, A. P.; Harris, L. G.; Horskotte, M. A.; Knobloch, J. K.-M.; Ragunath, C.; Kaplan, J. B.; Mack, D. Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials* **2007**, *28*, 1711−1720.
(23) Jakubovics, N. S.; Grant Burgess, J. Extracellular DNA in oral microbial biofilms. *Microbes. Infect.* **2015**, *17*, 531−537.
(24) Vilain, S.; Pretorius, J. M.; Theron, J.; Brözel, V. S. DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms. *Appl. Environ. Microbiol.* **2009**, *75*, 2861−2868.
(25) Gödeke, J.; Heun, M.; Bubendorfer, S.; Paul, K.; Thormann, K. M. Roles of two *Shewanella oneidensis* MR-1 extracellular endonucleases. *Appl. Environ. Microbiol.* **2011**, *77*, 5342−5351.
(26) Ripoll-Rozada, J.; Zunzunegui, S.; De La Cruz, F.; Arechaga, I.; Cabezón, E. Functional interactions of VirB11 traffic ATPases with VirB4 and VirD4 molecular motors in type IV secretion systems. *J. Bacteriol.* **2013**, *195*, 4195−4201.
(27) Grohmann, E.; Christie, P. J.; Waksman, G.; Backert, S. Type IV secretion in Gram-negative and Gram-positive bacteria. *Mol. Microbiol.* **2018**, *107*, 455−471.
(28) Low, H. H.; Gubellini, F.; Rivera-Calzada, A.; Braun, N.; Connery, S.; Dujeancourt, A.; Lu, F.; Redzej, A.; Fronzes, R.; Orlova, E. V.; Waksman, G. Structure of a type IV secretion system. *Nature* **2014**, *508*, 550−553.
(29) Defrancesco, A. S.; Masloboeva, N.; Syed, A. K.; DeLoughery, A.; Bradshaw, N.; Li, G.-W.; Gilmore, M. S.; Walker, S.; Losick, R. Genome-wide screen for genes involved in eDNA release during biofilm formation by *Staphylococcus aureus*. *Proc. Natl. Acad. Sci.* **2017**, *114*, e5969−e5978.
(30) Jurcisek, J. A.; Brockman, K. L.; Novotny, L. A.; Goodman, S. D.; Bakaletz, L. O. Nontypeable *Haemophilus influenzae* releases DNA and DNABII proteins via a T4SS-like complex and ComE of the type IV pilus machinery. *Proc. Natl. Acad. Sci.* **2017**, *114*, E6632−E6641.
(31) Jiang, Y.; Qian, F.; Yang, Y.; Liu, Y.; Dong, F.; Xu, C.; Sun, B.; Chen, B.; Xu, X.; Li, Y.; Wang, R.; Yang, S. CRISPR-Cpf1 assisted genome editing of *Corynebacterium glutamicum*. *Nat. Commun.* **2017**, *8*, 15179.
(32) Liu, D.; Chen, Y.; Li, A.; Ding, F.; Zhou, T.; He, Y.; Li, B.; Niu, H.; Lin, X.; Xie, J.; Chen, X.; Wu, J.; Ying, H. Enhanced butanol production by modulation of electron flow in *Clostridium acetobutylicum* B3 immobilized by surface adsorption. *Bioresour. Technol.* **2013**, *129*, 321−328.
(33) Lan, T. Q.; Wei, D.; Yang, S. T.; Liu, X. Enhanced cellulase production by *Trichoderma viride* in a rotating fibrous bed bioreactor. *Bioresour. Technol.* **2013**, *133*, 175−182.
(34) Yang, L.; Zheng, C.; Chen, Y.; Ying, H. *FLO* genes family and transcription factor *MIG1* regulate *Saccharomyces cerevisiae* biofilm formation during immobilized fermentation. *Front. Microbiol.* **2018**, *9*, 1860.
(35) Horton, R. M. PCR-mediated recombination and mutagenesis. SOEing together tailor-made genes. *Mol. Biotechnol.* **1995**, *3*, 93−99.
(36) Yu, B.; Zhang, X.; Sun, W.; Xi, X.; Zhao, N.; Huang, Z.; Ying, Z.; Liu, L.; Liu, D.; Niu, H.; Wu, J.; Zhuang, W.; Zhu, C.; Chen, Y.; Ying, H. Continuous citric acid production in repeated-fed batch fermentation by *Aspergillus niger* immobilized on a new porous foam. *J. Biotechnol.* **2018**, *276-277*, 1−9.
(37) Chen, T.; Liu, N.; Ren, P.; Xi, X.; Yang, L.; Sun, W.; Yu, B.; Ying, H.; Ouyang, P.; Liu, D.; Chen, Y. Efficient biofilm-based fermentation strategies for l-threonine production by *Escherichia coli*. *Front. Microbiol.* **2019**, *10*, 1773.
(38) Liu, L.; Yu, B.; Sun, W.; Liang, C.; Ying, H.; Zhou, S.; Niu, H.; Wang, Y.; Liu, D.; Chen, Y. Calcineurin signaling pathway influences *Aspergillus niger* biofilm formation by affecting hydrophobicity and cell wall integrity. *Biotechnol. Biofuels.* **2020**, *13*, 54.
(39) Cesarone, C. F.; Bolognesi, C.; Santi, L. Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Anal. Biochem.* **1979**, *100*, 188−197.
(40) Thompson, C. T.; Dvorak, J. A. Quantitation of total DNA per cell in an exponentially growing population using the diphenylamine reaction and flow cytometry. *Anal. Biochem.* **1989**, *177*, 353−357.
(41) Jurcisek, J. A.; Bakaletz, L. O. Biofilms formed by nontypeable *Haemophilus influenzae* in vivo contain both double-stranded DNA and type IV pilin protein. *J. Bacteriol.* **2007**, *189*, 3868−3875.
(42) Qin, Z.; Ou, Y.; Yang, L.; Zhu, Y.; Tolker-Nielsen, T.; Molin, S.; Qu, D. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. *Microbiology* **2007**, *153*, 2083−2092.

https://dx.doi.org/10.1021/acsomega.0c05095
ACS Omega 2020, 5, 33314−33322