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

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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-ΔexeM, 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. Biofilms have some "social" attributes such as aggregate growth, adhesive medium, and quorum sensing. Many strains on Earth can exist in the form of biofilms like Clostridium acetobutylicum, Escherichia coli (E. coli), Bacillus subtilis, and Saccharomyces cerevisiae. 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. Recently, researchers have discovered that cells in the biofilm could grow steadily with high activity, providing favorable conditions for industrial fermentation. Moreover, cells covered by biofilms could tolerate more harsh conditions such as high osmotic pressure, oxygen limitations, and high cell density during fermentation. 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. Furthermore, the biofilm cells that attach to the carrier surface could be renewed when fermentation broth is replaced with a fresh medium. 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.

In biofilms, eDNA plays an important role in the connection among the carriers, cells, extracellular polysaccharides, and proteins. eDNA was shown to have a great influence on the biofilm structure of Streptococcus mutans and Staphylococcus aureus. Sumei Liao et al. 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 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. So, eDNA was not only the key to biofilm formation but also the main component maintaining biofilm structure. Therefore,
an increase in eDNA accumulation would be beneficial to biofilm formation. Extracellular nuclease is one of the factors responsible for the degradation of eDNA.\textsuperscript{20} Researchers have shown that the removal of nuclease genes \textit{exeM} and \textit{exeS} promoted the accumulation of eDNA and biofilm formation in \textit{Shewanella oneidensis} (\textit{S. oneidensis}) MR-1.\textsuperscript{25} On the other hand, many bacteria have a type IV secretion system (T4SS) that could secret DNA or DNA-protein complexes.\textsuperscript{26,27} Within the T4SS, the \textit{VirB11} family proteins were the most important for the DNA and protein to go through.\textsuperscript{28−30} Although there was no research on \textit{exeM} or \textit{VirB11} in \textit{Corynebacterium glutamicum} (\textit{C. glutamicum}), in this study, we identified two genes highly homologous to \textit{exeM} and \textit{VirB11} in \textit{C. glutamicum} ATCC 13032-ProB, which produces l-proline.\textsuperscript{31} Subsequently, we engineered the \textit{C. glutamicum} ATCC 13032-ProB strain to enhance biofilm formation by removing \textit{exeM} and over-expressing \textit{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.\textsuperscript{17} Thus, 10 \(\mu\)L of DNase I (5 U) was added to each well in order to reduce the DNA content within \textit{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.\textsuperscript{17} The cells in biofilms were stained with DAPI and detected by a CLSM (Figure 1B). The morphological structure of the biofilm was formed on a 6-well plate after 72 h of incubation at 30 °C. Experiments were carried out in 96-well plates, and biofilm was quantified after 72 h of incubation at 30 °C.

![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 \(\mu\)L) (5 U) and 20 \(\mu\)L of seed culture were added to each well of a 96-well plate with 180 \(\mu\)L 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.](https://dx.doi.org/10.1021/acsomega.0c05095)

![Figure 2. EPS matrix of \textit{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 \textit{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 \(\mu\)g/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.](https://dx.doi.org/10.1021/acsomega.0c05095)
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-ΔexeM1, Pro-ΔexeM) 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-ΔexeM1, Pro-ΔexeM, and Pro-VirB11 were 16.3, 23.2, 27.4, and 25.2 μg/mL, respectively. Pro-ΔexeM 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-ΔexeM was increased by 75% compared with the original strain (2.17 vs 1.24), while Pro-ΔexeM1 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-ΔexeM 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-ΔexeM1 (Figure 4B), Pro-ΔexeM (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-ΔexeM (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 reconstruction of the eDNA staining of Pro-ΔexeM 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-ΔexeM strain showed a greater ability in its biofilm formation. Thus, the Pro-ΔexeM strain was chosen for immobilized repeated-batch fermentation.

L-Proline production in the first four batches had improved gradually in Pro-ΔexeM (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
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-\(\Delta\)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/\(\mu\)L) 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-\(\Delta\)exeM1 strain has removed one exeM gene or two exeM genes. However, we removed all exeM genes in the Pro-\(\Delta\)exeM strain.

Furthermore, the Pro-\(\Delta\)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-\(\Delta\)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-\(\Delta\)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.
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...
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-\(\Delta\)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.

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 to obtain a strain named Pro-\(\Delta\)exeM. 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-\(\Delta\)exeM. 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-\(\Delta\)exeM, Pro-\(\Delta\)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\(_4\))\(_2\)SO\(_4\), 0.5 g/L MgSO\(_4\), 7H\(_2\)O, 1 g/L KH\(_2\)PO\(_4\), and 2 g/L urea. The fermentation medium contained 100 g/L glucose, 20 g/L corn steep liquor, 30 g/L (NH\(_4\))\(_2\)SO\(_4\), 0.4 g/L MgSO\(_4\), 7H\(_2\)O, 1.2 g/L KH\(_2\)PO\(_4\), 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),

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

| strains          | relevant characteristics                  | source                  |
|------------------|-------------------------------------------|-------------------------|
| C. glutamicum ATCC 13032-ProB<sup>4</sup> | 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-\(\Delta\)exeM | C. glutamicum ATCC 13032-ProB with deleted part exeM | this study               |
| Pro-\(\Delta\)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               |
| pXMJ19-exeM     | pk18mobsacB with \(\Delta\)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                    |

<sup>4</sup>A gift from Prof. Sheng Yang (Institute of Plant Physiology & Ecology, CAS, Shanghai, China).
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 flask was 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/cm³ 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 foam 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 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.

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### Table 2. Main Primers Used in This Study

| primers   | relevant characteristics                                      |
|-----------|--------------------------------------------------------------|
| exeM1-up-F | TCACCTGGCAGGACTCTCAACTC |
| exeM1-up-R | aagccagaaggaggggttaCACAGACAATTTGGTCGTGGTG |
| exeM1-down-F | ccagtaattgtagctctgccgactac |
| exeM1-down-R | TGCTGGCAGGCAGTCGCAATG |
| Cm-exeM1-F | GTTGGCAGGGTCGCCAATG |
| Cm-exeM1-R | CGAGACCTGCGGAGGGACGC |
| exeM-up-F | ACCACAAAAATTGGTCGTGGTGAC |
| exeM-up-R | CAGACCTGCGGAGGGACGC |
| exeM-down-F | GCTTGGCAGGCAGTCGCAATG |
| exeM-down-R | GCTTGGCAGGCAGTCGCAATG |
| VirB11-F | GCTTGGCAGGCAGTCGCAATG |
| VirB11-R | GCTTGGCAGGCAGTCGCAATG |
| Y-exeM-F | CAGACCTGCGGAGGGACGC |
| Y-exeM-R | GCTTGGCAGGCAGTCGCAATG |
| Y-VirB11-F | GCTTGGCAGGCAGTCGCAATG |
| Y-VirB11-R | GCTTGGCAGGCAGTCGCAATG |

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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, while 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.

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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.

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