Isocitrate dehydrogenase of *Bacillus cereus* is involved in biofilm formation

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**Abstract**

Isocitrate dehydrogenase (IDH), a key enzyme in the TCA cycle, participates in the formation of biofilms in *Staphylococcus aureus*, but it remains to be clarified whether it is involved in the formation of *Bacillus cereus* biofilms. In this study, we scanned the genome of *B. cereus* 0-9 and found a gene encoding isocitrate dehydrogenase (FRY47_22620) named *icdH*. The IcdH protein was expressed and purified. The enzyme activity assay showed that the protein had IDH activity dependent on NADP⁺, indicating that this gene encoded an IDH. The ΔicdH mutant and its complemented strains were obtained by a homologous recombination strategy, and crystal violet data and CLSM were measured. The results showed that the biofilm yield of the mutant ΔicdH decreased, and the biofilm morphology also changed, while the growth of ΔicdH was not affected. The extracellular pH and citric acid content results showed that the ΔicdH mutant exhibited citric acid accumulation and acidification of the extracellular matrix. In addition, the addition of excess Fe³⁺ restored the biofilm formation of the ΔicdH mutant. It is speculated that IDH in *B. cereus* may regulate biofilm formation by modulating intracellular redox homeostasis. In addition, we found that the *icdH* deletion of *B. cereus* 0-9 could result in a reduced sporulation rate, which was significantly different from sporulation in *B. subtilis* caused by interruption of the stage I sporulation process due to *icdH* loss. All the above results provide us with new insights for further research on IDH.

**Keywords** *Bacillus cereus* · Biofilm · TCA cycle · IDH

**Introduction**

During long-term evolution, in response to a variety of environmental stresses, many *Bacillus* species have developed various adaptations and adopted protective strategies to improve their survival ability by sensing changes in the conditions inside and outside the cell, such as swarming motility, metabolic enzyme production, and biofilm formation (Bais et al. 2004). Biofilms are special structured bacterial communities with highly organized structures and matrix components (Branda et al. 2001). These cell communities are bound together mainly by a self-secreted extracellular matrix composed of exopolysaccharide and protein, which are biosynthesized via the products encoded by the operon *epsA-O* (henceforth simply *eps*) and the terminal gene *tasA* of the *yqXM-sipW-tasA* operon (henceforth simply *sipW*), respectively (Branda et al. 2006 and Frances et al. 2008). Biofilms are capable of increasing adhesion on biotic and abiotic surfaces, as well as resisting several kinds of environmental stresses, including nutrient deficiency and oxidative stress, which enhance plant root colonization and biological control efficiency (Ilana et al. 2013). Studies have shown that the formation of biofilms is a very complex process, and the environmental impact is especially important in this process, such as the pH of the environment and the nutrient composition, temperature, osmotic pressure, iron concentration and surface characteristics of the contact medium (Tang et al. 2006). In the natural environment, more than 90% of microorganisms exist in the form of biofilms. Research on biofilms has become popular. For instance, Tantan, Gao et al. (2019)
proposed that the ptsH gene influences biofilm formation; Prasad et al. (2015) suggested that phosphorylation controls biofilm formation.

Nitrogen and carbon sources are essential for the survival of bacteria, while the TCA cycle, also known as the Krebs cycle, is a ubiquitous metabolic pathway in aerobic organisms (Lynne et al. 2014). Various important intermediates formed in the pathway provide a large number of biosynthetic carbon sources. In addition, the thioester bond of succinyl-CoA generated by the two oxidative decarboxylation steps of the TCA cycle hydrolyses under the action of succinate thiokinase and generates a large amount of ATP, which provides energy for the activities of living organisms. This is the final metabolic pathway for three major nutrients (sugars, lipids and amino acids), crucial for their metabolism and transformation, and for the reoxidation of fermentation products, which impacts the living environment of bacteria. IDH is an important enzyme in the TCA cycle that can catalyse the oxidative decarboxylation of isocitrate to form CO₂, H₂O, and α-ketoglutarate and reduce NAD⁺ or NADP⁺ to NADH or NADPH, respectively (Zhang et al. 2018). According to the spatial characteristics, IDH is generally classified into two types; namely, NAD-IDH and NADP-IDH. IDH is also divided into two types according to the degree of molecular polymerization: monomers, with molecular weights of approximately 40–50 kD, and homodimers, with molecular weights of approximately 80–100 kD (Kaori 2016). Both monomeric IDH and dimeric IDH play vital roles in bacterial biosynthesis, energy metabolism, and antioxidant stress. IDH is also a critical intersection point between the TCA cycle and the glyoxylate shunt. The reversible phosphorylation of NADH-dependent IDH can switch the two pathways by changing carbon flow. Furthermore, isocitrate can be catalytically converted to glyoxylate and succinate, and IDH plays an important role in the glyoxylate shunt (Lv 2017).

It has been reported that in S. aureus, IDH is involved in the formation of biofilms by regulating the redox state in vivo (Ilana et al. 2013). However, the role of IDH in the TCA cycle in biofilm formation of B. cereus remains unknown. We studied the effect of IDH on biofilm formation in B. cereus 0-9 (GenBank: CP042874.1). B. cereus 0-9 is a gram-positive endophytic bacterium that was isolated from wheat roots in our laboratory, is easily cultured, can form strong biofilms and generate spores in harsh environments, and exhibits favourable swimming ability. In our published papers, B. cereus 0-9 was shown to have the ability to control wheat sheath blight, exhibiting potential application value and good control effects. In this work, we investigated the relationship between IDH from the TCA cycle and biofilms based on gene knockout and complementation, protein induction and purification, enzyme activity determination and other techniques.

Materials and methods

Strains, plasmids, media, and growth conditions

The strain B. cereus 0-9 was obtained from the China Center for Type Culture Collection (CCTCC No.: M209041) and is the subject of a patent that has been approved in China (Patent No.: 200910064867.2). E. coli BL21 (DE3), E. coli 116 (pir +) and E. coli GM2163 (dam-) were purchased from Epicentre. The plasmids PMAD-T7, PMADCHI, PET28a and pAD123-pgal were constructed and saved by this laboratory. The plasmid PMAD-T7 was used to construct the mutant strains, and the plasmids PMADCHI and pAD123-pgal were used for the construction of complemented strains. E. coli 116 and GM2163 were used for cloning genes, and E. coli BL21 (DE3) was used for protein expression. All strains were stored in LB medium containing 25% glycerol at −80 °C before use. B. cereus 0-9 and its derivative strains are usually cultured at 30 °C in Luria–Bertani (LB) liquid medium (1% peptone, 1% NaCl, 0.5% yeast extract, pH 7.0) or in solid LB medium (LB liquid medium supplemented with 1.6% agar). E. coli BL21 (DE3), E. coli 116 (pir +) and E. coli GM2163 (dam-) were incubated at 37 °C in liquid or solid LB. When necessary, antibiotics were added to the culture medium at the following concentrations: 3 µg/ml erythromycin for B. cereus growth and 100 µg/ml ampicillin and 50 µg/ml kanamycin for E. coli growth (Karen et al. 2013). In the biofilm formation experiment, LBS medium (1% tryptone, 0.5% yeast medium, 0.5% NaCl, 0.1% sodium citrate, 0.2% ammonium sulfate, 0.02% MgSO₄·7H₂O, 1.4% K₂HPO₄, 0.6% KH₂PO₄, and 0.1% glucose, (w/v)) was used. In the spore formation experiment, EPS medium (0.7% K₂HPO₄, 0.3% KH₂PO₄, 0.01% MgSO₄·7H₂O, 0.001% CaCl₂, 0.0001% FeSO₄, 0.01% NaCl, 0.1% glucose, and 0.02% yeast medium, (w/v)) was used.

Strain construction

The icdH gene (locus: FRY47_22620) deletion mutant strain (ΔicdH) in B. cereus 0-9 was constructed using a homologous recombination strategy. The two fragments for allelic exchange that were amplified with primeSTAR polymerase (purchased from Takara company) from B. cereus 0-9 genomic DNA using primers were digested with the corresponding restriction endonuclease enzyme and then inactivated in a metal bath at 80 °C for 10 min. The two fragments were ligated into pMAD-T7 in a corresponding double enzyme digestion. The pMAD-based plasmid constructs were transferred into the E. coli 116...
strain by the heat shock transformation method (Sha et al. 2011). To verify the correctness of the cloned DNA fragment, plasmids were digested by enzymes and amplified by PCR. The plasmid containing the correct DNA fragment was transferred into \( E. coli \) GM2163 by electroporation (1.2 kV, Bio–Rad) (Lu et al. 2012). After being purified again, the plasmid was transformed into \( B. cereus \) 0-9 competent cells. After culturing at 30 °C for 1 h at 100 rpm, the cells were spread on LB agar plates. After incubation at 30 °C overnight, the plates were screened for blue and white colonies, and the correct clones were obtained. All the derivative strains of \( B. cereus \) 0-9 were constructed according to the method described above. All primers are listed in Table 1.

**Growth curve analysis**

The wild-type strain \( B. cereus \) 0-9 and its derivatives were cultured overnight in LB broth. These cultures were transferred to fresh LB liquid medium and incubated with shaking at 30 °C and 220 rpm until the \( \text{OD}_{600} \) was approximately 0.5. Fifty microlitres of the cultures with the same \( \text{OD}_{600} \) were inoculated into 2 ml of fresh LB liquid medium. Each sample examination was repeated five times. LB medium was used as a growth control. The \( \text{OD}_{600} \) was measured in Bioscreen C (Oy Growth Curves Ab Ltd, Finland) at 30 °C at 30-min intervals. The \( \text{OD}_{600} \) value of each micropore was measured every 30 min, and the growth curve of the tested strains was recorded automatically. Growth curves of the wild-type strain \( B. cereus \) 0-9 and its derivatives in LBS liquid medium were determined following the above procedure.

**Analysis of biofilm formation**

For analysis of solid surface-associated biofilm formation, the crystal violet staining method with some modifications was used (Sinem et al. 2018 and Si et al. 2016). A single colony of \( B. cereus \) 0-9 and its mutant strain was inoculated into 5 ml of LB medium and incubated at 30 °C overnight. Approximately 50 μl of the overnight culture was inoculated into 2 ml of LBS medium in glass culture tubes with a diameter of 0.8 cm. The tubes were incubated in an upright position at 30 °C for 5 day before surface pellicles and cultures were carefully removed from the tubes. The remaining cells and matrices in each tube were stained with 2.5 ml of 0.1% (w/v) crystal violet solution for 20 min at 25 °C. After washing three times with distilled water, the crystal violet attached to the biofilm was solubilized in 2.5 ml of mixed eluent (ethanol:propanone = 7:3, (v/v)). Two hundred microlitres of the solution were quantified by measuring the absorbance at 570 nm. Each sample examination was repeated three times. The same procedure was used for 24-well polystyrene board testing of biofilms.

| Primer | Sequence (5′→3′end) |
|--------|---------------------|
| icdH-up-BamHI-s | ACACGATCCCGCTTGTACTTCATGACATCA |
| icdH-up-XhoI-a | CACACCGGGTACTATTACAACACCATAG |
| icdH-d-XhoI-s | ACACCTCGAGATTTAAAACATGGATGGCAG |
| icdH-d-EcoRI-a | CACAGAATTCTCATTTCAACTAAAGAAGCTC |
| icdHpro-lap-MluI-s | ACACACCGGATTAGAAATTTTGAAGAAATAC |
| icdHpro-lap-a | GTCAAGTGTGAAAACCCCTGACATTTTCTCATTTTTTGAAAAATTC |
| icdHorf-lap-s | CAGAAAGGGAGAGAATGTGATGAGATTTACATGACGAC |
| icdHorf-lap-XhoI-a | CACACTCGAGTTATGCGTTTTTGTATTTCGATTAC |
| PicdH-EcoRI-s | ACACGATTCGAGTAAGAAATTTTGAAGATTC |
| PicdH-overlap-a | ATTTTTTCACCTTGGCCACATGACATTTCTTTTTCTGAG |
| 168icdH-overlap-a | GAAAAAGGAGAAGATGTCAATGGTTGAGCAAAAGGTGAACAAATTAG |
| 168icdH-XhoI-a | CACACTCGAGTTATGCGTTTTTGTATTTCGATTAC |
| icdHcom-XhoI-a | CACACTCGAGTATGCGTTTTTGTATTTCGATTAC |
| icdHorf-28a-BamHI-s | ACAGAGATCCATGACAGAGTTGAAAAAATTACTG |
| gfp-XhoI-a | CACACTCGAGTTATGCGTTTTTGTATTTCGATTAC |
| PsipW-BamHI-s | CACAGGATCCGGAAGAATGAAATGTTGGGA |
| PsipW-gfp-a | AGTCTTCTCTTTTACTATGATCGTCTCTCCTCCGTGTTTTTA |
| gfp-PsipW-s | AACGGAGAGGGAGAGAGAGAGATGAGTAAAGGAGAAGAACTTTTAC |
| cdlHcom-XhoI-a | CACACTCGAGTATGCGTTTTTGTATTTCGATTAC |
| icdHorf-28a-BamHI-s | ACACCGATCCATGACAGAGTTGAAAAAATTACTG |
Confocal laser scanning microscopy (CLSM) analysis

The biofilms were observed by confocal laser scanning microscopy as previously described with some modifications (Olympus FV1000) (Bayjuinov et al. 2018). The biofilm culture method referred to the above method. The difference was that approximately 50 μl of overnight culture, which was adjusted to the same OD600 value, were inoculated into 2 ml of LBS medium in a glass-bottom cell culture dish (Cat. No: 801002; φ15 mm, purchased from Benxin Biology). The cell culture dishes were statically cultured in a horizontal position at 30 °C for 5 day. Each sample examination was repeated five times. Finally, the LBS medium was slowly removed with a pipette (Gao et al. 2015). As the liquid itself contained expressed fluorescent proteins, the formation of biofilms could be monitored by CLSM through the bottom of the cell culture dish. The biofilm images were analysed by ZEN lite software (blue edition). Images were obtained from random positions on the biofilms formed on the cell culture dish. Confocal stack images were used to determine the thickness of the biofilms.

Protein overexpression and purification

The construction of icdH expression vectors and protein purification were performed as described in our published article with some modifications. The open reading frame of the icdH gene was amplified from B. cereus 0-9 genomic DNA. The correct fragment that had been verified by sequencing was ligated with the pET28a vector to obtain a recombinant plasmid. For verification by double enzyme digestion, the recombinant plasmid was transformed into E. coli BL21 (DE3). The recombinant IcdH protein was expressed in the E. coli BL21 (DE3) strain according to a previously described method (Diethmaier et al. 2014). When E. coli BL21 (DE3) cells harbouring the expression plasmid were cultivated to logarithmic phase, 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was added for induction for 4 h at 37 °C. The cells were harvested by centrifugation and lysed by sonication (Hayashi et al. 2014). After lysis, the crude extracts were centrifuged at 4548×g for a minimum of 60 min.

Nickel-immobilized metal affinity chromatography using a His-Trap column was used for IcdH protein purification. The bound protein molecules were eluted by imidazole with the following elution procedure: first, the proteins were eluted with 10 ml of imidazole buffer and were then eluted with 10 ml of 40, 60, 80, 100, 200, and 500 mM imidazole buffer. Each eluent fraction was collected and detected using SDS–PAGE electrophoresis for the distribution of IcdH protein (Kobayashi and Takada 2014). The eluted proteins were collected, and the eluent was then dialyzed overnight in PBS to remove imidazole. The purity of IcdH was estimated to be > 90% by SDS–PAGE. The purified recombinant protein was stored at ~ 20 °C.

Assay for IDH enzyme activity

IDH enzyme activity was carried out as reported previously with some modifications (Ochiai et al. 1979). The enzyme activity of IDH was evaluated at 25 °C in 96-well microplates. One milliliter of enzyme activity reaction mixture contained 20 mM Tris–HCl buffer (pH 8.0), 1.0 mM DL-isocitrate, 2 mM MnCl2, and 1.0 mM NADPH. An appropriate amount of enzyme was added to the reaction mixture at the initial reaction stage, and 200 μl of reaction mixture was added to 96-well microplates. The NADPH curve was detected at 340 nm with a thermostated Cary 300 UV–vis spectrophotometer (Varian, USA). The molar extinction coefficient was 6.22 mM−1·cm−1. The protein concentrations were monitored with a Bio–Rad Protein Assay Kit (Bio–Rad, USA). Bovine serum albumin was used as a standard. The final data were the average of at least three duplicates. The amount of enzyme that reduced 1 μmol of NADPH per minute was considered one unit of enzyme activity.

Determination of citric acid

The citric acid content was determined using a citric acid content determination kit purchased from Suzhou Keming Biotechnology Company Limited. (www.Cominbio.com).

Determination of spore formation

The spore ratios of the wild-type strain B. cereus 0-9 and its derivatives were determined by the spread plate technique, which had been published previously, with some modifications (Luisa et al. 2015 and Huang et al. 2021). The overnight culture solution with the same OD600 was inoculated into EPS liquid medium at 1% (v/v). Then, the culture was incubated at 37 °C with shaking at 220 rpm. After 31 h, 1 ml of each sample suspension was serially diluted, and 100 μl of each dilution was plated on solid LB medium for total cell density determination in CFU/ml. At the same time, for spore cell density, each serially diluted sample was immediately heated at 80 °C for 10 min, and then, 100 μl of heat-treated bacterial solution was uniformly coated onto an LB solid plate. The plates were cultured at 30 °C overnight, and the total cells and spore cell densities were counted. The sporulation efficiency (%) was calculated as the ratio between spore cell and total cell densities.
**Determination of florescence intensity**

The fluorescence intensity of the strain was determined according to a previously reported method (Saeid et al. 2016). The *B. cereus* strain 0-9 and its mutant strain containing the *sipW*-GFP reporter gene were incubated overnight in 5 ml of LB medium at 30 °C with shaking at 220 rpm (Thimmaraju et al. 2007). Approximately 1 ml of overnight culture was inoculated into 100 ml of LBS medium and incubated at 30 °C with shaking at 220 rpm. After 24 h, the cells were collected by centrifugation at 4 °C and 13,523 g, and then suspended in 1 ml of ddH₂O. Suspensions with the same OD₆₀₀ value were added to 96-well microplates and examined with a microporous plate-type multifunctional detector (blue, 490 nm). The normalized fluorescence intensity was determined as the ratio of fluorescence intensity to OD₆₀₀. Each data point represents the average value of three independent experiments.

**Results**

**Deletion of the icdH gene decreases the biofilm formation of *B. cereus* 0-9**

There have been many reports on the role of the TCA cycle in *Bacillus* spp. For example, Guan et al. (2018) proposed the effects of the TCA cycle on the growth and pectinase activity of *Bacillus licheniformis* DY2; Liu et al. (2018) showed that the TCA cycle regulated bacitracin production in *Bacillus licheniformis* DW2. However, reports on the role of isocitrate dehydrogenase in *B. cereus* have been relatively scarce until now. Therefore, *icdH*, an IDH-encoding gene from *B. cereus* 0-9, was knocked out according to the above described double-exchange methods, and the mutant strain Δ*icdH* was successfully constructed. The bacterial biofilm observation experiment was carried out in LBS medium. Fifty microlitres of fresh bacterial liquid with an OD₆₀₀ value of approximately 0.7 was added to 2 ml of LBS medium, and the culture conditions were as described in “Analysis of biofilm formation”. The biofilm results of the strains are listed in Fig. 1. It was clearly observed that the differences in biofilm formation in LBS medium were particularly significant when comparing Δ*icdH* and *B. cereus* 0-9. Under the same conditions, the amount of biofilm of the mutant strain Δ*icdH* was greatly reduced compared to *B. cereus* 0-9, and the results are listed in Fig. 1A. To further confirm this result, the biofilm formation amount of the strains was measured through crystal violet staining, and the results are shown in Fig. 1B. The amount of mutant strain Δ*icdH* showed a 50% decrease in biofilm formation compared to *B. cereus* 0-9. The above results indicated that *icdH* gene deletion decreased the biofilm formation of *B. cereus* 0-9.

![Fig. 1](image)

**At the same time, the microplate-based method was used to observe the phenotype of the solid biofilms. Polystyrene 24-well microplates were used in this study (Φ, 15.6 mm, purchased from Corning). When grown in LBS medium, *B. cereus* 0-9 formed a large amount of floating biofilms, which were closely connected to the surface of the petri dish to form a complete overall floating object that was dense and thick. However, the mutant strain Δ*icdH* produced a small amount of dispersed biofilms floating on the surface of the dish (Fig. 2). The biofilms produced by *B. cereus* 0-9 and the mutant strain Δ*icdH* on the microporous plates were quantitatively analysed by a crystal violet staining assay. The results were consistent with the above results. The mutant strain Δ*icdH* showed less biofilm formation than *B. cereus* 0-9, displaying only 50% of the biomass of *B. cereus* 0-9 biofilms. The above results showed that *icdH* gene deletion decreased the biofilm formation of *B. cereus* 0-9. These results reconfirmed that the *icdH* gene influenced the biofilm formation of *B. cereus* 0-9.**
Deletion of the icdH gene did not affect the growth of B. cereus 0-9 in LB or LBS liquid medium

These results suggested that the absence of the icdH gene affected the biofilm formation of B. cereus 0-9. We first speculated whether the deletion of the icdH gene caused the growth defect of the mutant strain ΔicdH, which might affect the biofilm formation of B. cereus 0-9. Therefore, under the same culture conditions, the growth of B. cereus 0-9 and the mutant strain ΔicdH were measured in LB liquid medium. The test procedure was described above in “Growth curve analysis”. The measured results of the growth curve are shown in Fig. 3. The results in Fig. 3A show that the growth trends of the mutant strain ΔicdH and B. cereus 0-9 were not significantly different in either the logarithmic period or the stable period when cultured in LB medium. Considering the difference in composition between LB medium and LBS medium, the growth curves of the mutant strain ΔicdH and B. cereus 0-9 in LBS liquid medium were measured, and the results are shown in Fig. 3B. Similarly, the growth trend of the mutant strain ΔicdH and B. cereus 0-9 in LBS medium was consistent with the results in LB medium, and the growth differences between the mutant strains ΔicdH and B. cereus 0-9 could be ignored. Therefore, the difference in biofilm formation due to the influence of icdH gene deletion on the normal growth of B. cereus 0-9 was excluded.

Complementation of the icdH gene restored biofilm formation of the mutant to the wild-type level

From the above biofilm measurement results, it was clear that under the same conditions, when cultured in LBS medium, the amount of biofilm formed by ΔicdH was much lower than that of B. cereus 0-9. Therefore, we wondered whether deletion of the icdH gene directly affected the biofilm formation of B. cereus 0-9. The promoter and open reading frame of the icdH gene were obtained from the B. cereus 0-9 genome by amplification, and the complemented strain ΔicdH::icdH was constructed following the above method. According to the biofilm formation assay, the biofilm of the complemented strain ΔicdH::icdH was measured, and the results are shown in Fig. 1A. As we expected, the biofilm formation of the complemented strain ΔicdH::icdH was approximately the same as that of B. cereus 0-9 in LBS medium, which recovered the B. cereus 0-9 levels. At the
same time, crystal violet staining data of the complemented strain ΔicdH::icdH were obtained and are shown in Fig. 1B. From the results, it could be clearly seen that the amount of biofilm formed by the complemented strain ΔicdH::icdH was essentially the same as that formed by B. cereus 0-9. The results showed that the complementation made up for the biofilm defects of the mutant strain ΔicdH.

We further verified whether loss of the icdH gene in B. cereus 0-9 caused a reduction in biofilm formation. The known IDH gene from the genome of B. subtilis BS168 was cloned using the primers Bs168icdH-overlap-s/Bs168icdH-a; this gene shared high identity with the gene from B. cereus 0-9, up to 84.65%. The complemented strain ΔicdH::icdHBS was constructed as described above. Biofilm formation experiments were also carried out in LBS medium. The result was the same as that of the complemented strain ΔicdH::icdH (Fig. 1A). The amount of biofilm formation of the complemented strain ΔicdH::icdHBS also recovered to the B. cereus 0-9 level. The crystal violet staining data of the complemented strain ΔicdH::icdHBS were similar to those of the complemented strain ΔicdH::icdH, which are shown in Fig. 1B. Similarly, the crystal violet staining data of the biofilm of the complemented strain ΔicdH::icdHBS were essentially equal to those of B. cereus 0-9.

In addition, considering that the IDH gene from the E. coli genome also shared 62.87% identity with the gene from B. cereus 0-9, the icdH gene fragment was amplified from the E. coli BL21 genome using the primers BL21icdH-s/BL21icdH-xhol-a. At the same time, the promoter of the icdH gene in B. cereus 0-9 was used to obtain the complemented strain ΔicdH::icdHBL through the strain construction method described previously. Similarly, the biofilm formation of the complemented strain ΔicdH::icdHBL in LBS liquid medium was measured, and crystal violet staining data of the biofilm were also obtained. The results are shown in supplementary material Fig. 1S and indicated that the complemented strain ΔicdH::icdHBL also recovered the biofilm formation level of B. cereus 0-9. The above results suggested, we preliminarily confirmed that the deletion of isocitrate dehydrogenase (icdH) of the TCA cycle in B. cereus 0-9 seriously affected the formation of biofilms.

The deletion of icdH decreased the expression of genes encoding biofilm components

To further demonstrate the effect of icdH on biofilms, the fluorescent transcription fusion reporter strains 0-9 (PsipW) and ΔicdH (PsipW) were obtained. The formation of biofilms produced by the mutant ΔicdH and B. cereus 0-9 strains could be quantitatively detected using a microporous plate-type multifunctional detector (green, 450 nm, GloMax Multi, USA). The fluorescence spectrophotometry results obtained after the strains were incubated in LBS medium for 10 day are listed in Table 2. Compared with the GFP reporter strain 0-9 (PsipW), the GFP fluorescence intensity of the mutant strain ΔicdH was only 43% that of the reporter strain 0-9 (PsipW). In addition, the absence of icdH also delayed the expression of genes related to the biofilm matrix. Under the same conditions, the GFP reporter strain 0-9 (PsipW) was detected for approximately 72 h under static culture conditions, while the mutant ΔicdH (PsipW) needed approximately 216 h. In oscillatory culture, the expression of sipW was detected in 0-9 (PsipW) for approximately 24 h, and the mutant ΔicdH (PsipW) needed approximately 72 h. This suggested that the icdH gene deletion decreased the expression levels of genes encoding biofilm components.

Table 2 The fluorescence intensity of transcription fusion strains 0-9 (P_sipW) and ΔicdH(P_sipW)

| Bacteria name         | Fluorescence intensity |
|-----------------------|------------------------|
| The B. cereus 0-9     | 7.923 ± 0.008          |
| The B. cereus 0-9 harboring GFP reporter | 8.621 ± 0.15 |
| The mutant ΔicdH harboring GFP reporter | 3.704 ± 0.15 |

Each data represents the average value of three independent experiments.

CLSM observation confirmed that icdH deletion changes the structure of biofilms

The biofilm morphologies of the transcription fusion strains 0-9 (P_sipW) and ΔicdH (P_sipW) were observed under a two-photon laser confocal microscope (Olympus FV1000) at 100-fold magnification after 120 h of culture in LBS medium, as shown in Fig. 4. Figure 4A and B show that the biofilms of the mutant strain ΔicdH that formed at the bottom of the plastic dish were noticeably thinner with numerous voids, similar to discrete units of bacteria. However, the B. cereus 0-9 strain harbouring the GFP reporter formed a thick layer of uniformly dense biofilms with porous channels but no obvious voids. Figure 4C and D show stratified scan diagrams of the biofilms formed by the strain 0-9 (P_sipW) and the mutant strain ΔicdH (P_sipW). The strain 0-9 (P_sipW) produced thicker biofilms than the mutant ΔicdH (P_sipW). The thickness of the biofilm produced by strain 0-9 (P_sipW) was approximately 23 µm, while that of the mutant strain was only 6.72 µm. In addition, the transcription fusion strains of the complemented strains ΔicdH::icdH (P_sipW) and ΔicdH::icdHBS (P_sipW) were constructed and observed under the same conditions. The results showed that the two complemented strains also recovered the structure and morphology of the biofilm of B. cereus 0-9, forming dense and thick biofilms (supplementary material Fig .3S). From the results, it was confirmed that icdH deletion could change the structure of biofilms.
The icdH-encoded protein is an isocitrate dehydrogenase

The protein-expressing ΔicdH strain was successfully obtained and then the IcdH protein induced and purified according to the above experimental method. As shown in Fig. 5, the pure protein was approximately 46 kD in size, and its amino acid sequence was obtained (supplementary material Table 1S). The protein concentration was measured with the BSA-Bradford analysis method (Silvério et al. 2012). The activity of the newly purified enzyme was tested for 1 h according to the method described above for the determination of IDH activity. When the purified protein concentration was 1.46 mg/ml, the average enzyme activity in an hour was 0.86 U/ml (after deduction of the blank control). However, the activity of the inactivated control protein at the same concentration was only 0.115 U/ml under the same conditions. This result was essentially consistent with that of the blank control, which was 0.114 U/ml. When the protein stock was diluted by a factor of 10, the enzyme activity was still 0.33 U/ml (blank control had been deducted). On the basis of the above results, we found that the IcdH protein had the activity of IDH from the TCA cycle. The enzyme activity remained stable for approximately 24 h. We confirmed that the missing gene in the mutant strain, icdH, was the IDH-encoding gene from the TCA cycle in B. cereus 0-9.

Fig. 4 Biofilm morphology diagrams of the transcription fusion strains 0-9 (P_{ sipW } ) and ΔicdH (P_{ sipW } ). a Monolayer scan diagram of Bacillus cereus 0-9 (P_{ sipW } ). b Monolayer scan diagram of the ΔicdH (P_{ sipW } ) c Stratified scan diagram of biofilms of strains 0-9 (P_{ sipW } ). d Stratified scan diagram of biofilms of strains ΔicdH(P_{ sipW } )
IDH deficiency blocks the TCA cycle and causes citric acid accumulation

The absence of icdH resulted in the downstream steps of the TCA cycle not proceeding normally, which would severely affect many metabolic processes in vivo. It is well known that one of the important functions of IDH is to catalyse the oxidative decarboxylation of isocitrate to produce keto glutarate and CO2. To verify this result, exogenous α-ketoglutarate acid at 0.001% was added to the mutant strain ΔicdH cultured in liquid LBS medium. As we expected, the biofilm formation of the mutant strain ΔicdH was largely restored to the level of B. cereus 0-9 (Fig. 6). The results fully demonstrated the importance of the icdH gene in the TCA cycle, and the deletion of the icdH gene resulted in the blockade of downstream routes, thus affecting the formation of biofilms in the strain.

We speculated that the absence of icdH also led to the accumulation of large amounts of upstream citric acid while blocking the normal downstream route of the TCA cycle. The accumulation of citric acid might result in a pH change in the culture environment. Therefore, the pH values of the B. cereus strain 0-9 and the mutant strain ΔicdH cultured statically in LB or LBS media for 4 day at 30 °C were tested. The results are listed in Fig. 7. As expected, whether cultured in LB or LBS medium, the medium of the mutant strain ΔicdH had a lower pH value, even though the LBS medium contained a buffer system. At the same time, whether cultured in LB or LBS medium, both complemented strains ΔicdH∷icdH and ΔicdH∷icdHBS recovered the pH value of B. cereus 0-9 (supplementary material Fig. 4S). To further confirm our hypothesis, the citric acid content in the medium was determined using a citric acid assay kit. Under the same conditions, the citric acid content of the B. cereus strain 0-9, which was statically cultured in LBS medium for 4 day at 30 °C, was 3.889 μM/L, while the citric acid content of the mutant strain ΔicdH was 11.944 μM/L. Thus, the citric acid content in the LBS medium in which the mutant strain ΔicdH was incubated was nearly three times as high as that in B. cereus 0-9 medium. The above results were completely consistent with our hypothesis. They showed that the icdH gene deletion in the TCA cycle led to the accumulation of citric acid, which changed the pH value of the culture environment and inhibited biofilm formation.
Supplementation with excess Fe³⁺ can restore biofilm formation in icdH mutants

The lack of metal ions in cells results in reduced cell viability and affects biofilm formation. Citric acid is a good chelating agent and can chelate metal ions. The accumulation of citric acid may lead to increased iron ion chelation, resulting in a rapid decrease in the concentration of metal ions in the medium. To explore this hypothesis, different concentrations of exogenous iron ions were added to the LBS medium. Under the same culture conditions, biofilm formation experiments of B. cereus strain 0-9 and the mutant strain ΔicdH were performed. The results are shown in Fig. 8. From the results, it can clearly be seen that the addition of exogenous iron ions at different concentrations led to different degrees of recovery defects in the formation of biofilms of the mutant strain ΔicdH.

Deletion of the icdH gene affects the sporulation of B. cereus 0-9

Sheng et al. (1997) proved that deletion of the Bacillus subtilis isocitrate dehydrogenase gene causes a block at Stage I of sporulation. When the icdH gene was knocked out in B. subtilis, the spore production rate of the mutant strain was only 0.005% (Sheng et al. 1997). Therefore, we wondered whether the result of spore formation when the icdH gene was deleted in the B. cereus 0-9 genome would be similar to that in B. subtilis. The sporulation of the mutant strain ΔicdH was tested as described in “Determination of spore formation” and the results are shown in Fig. 9. To our surprise, the spore production rate of the mutant strain ΔicdH was only second to that of B. cereus 0-9. Under the same culture conditions, the spore production rate of the mutant strain ΔicdH reached 63.63%. However, the sporulation rate of B. cereus 0-9 was only 80.54%. Since the previous complemented strains all recovered the level of biofilm formation of B. cereus 0-9, we wondered whether the complemented strains could also compensate for the sporulation defect of the mutant strain ΔicdH. Under the same conditions, sporulation experiments of the complemented strains were performed. Surprisingly, the sporulation results of the complemented strains also recovered the B. cereus 0-9 sporulation level. The sporulation results are shown in Fig. 9. Under the same experimental conditions, the sporulation rates of the complemented strains ΔicdH::icdH and ΔicdH::icdHBL reached 82.6% and 81.2%, respectively. In addition, the sporulation rates of the complemented strain ΔicdH::icdHBL were tested, and the results are shown in supplementary material Fig. 2S. Similarly, the sporulation rates of the complemented strain ΔicdH::icdHBL also recovered to the level of B. cereus 0-9, up to 83.76%. According to these results, the roles of the icdH gene in B. subtilis were very different from those in B. cereus.

Discussion

In this report, we found for the first time that the icdH gene from the TCA cycle of B. cereus 0-9 affected biofilm formation. Under certain conditions, ΔicdH, a mutant lacking the icdH gene, produced very little biofilm when cultured in LBS medium compared with B. cereus 0-9. First, it was speculated that loss of icdH might affect the normal growth of the strains, causing differences in biofilms. To test this hypothesis, the growth of B. cereus 0-9 and ΔicdH in LB and LBS media was monitored, but the growth curves of the two strains showed no differences.
Several studies have reported that a three-gene operon, $yqxM$-$sipW$-$tasA$ ($sipW$ for short), which shares the same promoter and is simultaneously transcribed, is required for biofilm formation of bacteria (Sheng et al. 1997 and Frances et al. 2006). It was hypothesized that when the $icdH$ gene was deleted, the biofilm yield of $B. cereus$ 0-9 would decrease, and the expression of operon $sipW$, which was essential for bacterial biofilm formation, would also decrease. Therefore, the GFP reporter strains 0-9 ($P_{sipW}$) and ΔicdH ($P_{sipW}$) were constructed and detected using a microporous plate-type multifunctional detector (green, 450 nm, GloMax Multi, USA) and a two-photon laser confocal microscope (Olympus FV1000). As we expected, after deletion of the $icdH$ gene, the expression of the $sipW$ operon decreased. Surprisingly, deletion of the $icdH$ gene not only led to a decrease in $B. cereus$ 0-9 biofilm yield but also changed the structure and morphology of biofilms. To date, there are many regulatory routes of bacterial biofilm formation that have been recognized by researchers. At present, we have not found any conclusive evidence on which regulatory route the $icdH$ gene is involved in $B. cereus$ 0-9 biofilm formation. In future experiments, we...
will look for further evidence that the icaD gene regulates biofilm pathways.

It is well known that in the TCA cycle, citric acid can be isomerized to form isocitric acid, which can form α-ketoglutaric acid under the action of IDH, after which the cycle continues its subsequent steps. It was hypothesized that the absence of icaD might cause the failure of conversion of isocitric acid to α-ketoglutaric acid. Then, different concentrations of exogenous α-ketoglutaric acid were directly added to the LBS medium. Consistent with our prediction, the mutant strain showed restoration of the level of biofilm production to that of  B. cereus  0-9 in LBS medium. The results fully demonstrated the importance of the icaD gene in the TCA cycle, and that the deletion of the icaD gene resulted in a blockage of downstream routes, thus affecting the formation of biofilms by the strain. In addition, it has been reported that most bacteria need a pH range of 7.0–8.0 to form biofilms (Ilana et al. 2013 and Gao et al. 2019). The accumulation of large amounts of citric acid may result in a change in pH in the culture environment and affect the formation of biofilms. The results of pH monitoring and citric acid content determination in the culture medium of  B. cereus  0-9 and ΔicaD fully indicated that damage to the icaD gene from the TCA cycle led to citric acid accumulation, which changed the pH value of the culture environment and inhibited biofilm formation.

Iron ions are important for the function of haeme-containing cytochromes, which play a crucial role in the respiratory organs of bacteria and thus affect the vitality of the cell (Gaballa et al. 2008). It has been shown in the literature that the bacterial cellular respiration chain affects the production of the bacterial biofilm matrix (Ilana et al. 2013). Iron ions are an important component of the respiratory system, and the concentration of iron ions affects the cellular respiration chain function of the bacteria; therefore, the concentration of iron ions also affects the production of the bacterial biofilm matrix (Sarah et al. 2018). The literature suggests that a high concentration of Fe³⁺ is beneficial to the production of the biofilm matrix of bacteria, thus promoting biofilm formation (Ilana et al. 2013). However, a low concentration of iron ions may cause the loss of pigment function in cells, leading to impaired cellular respiration, thus reducing biofilm matrix production and biofilm formation. After adding different concentrations of exogenous Fe³⁺ to the LBS medium, the recovery of biofilms generated by the ΔicaD strain, demonstrates that deletion of the icaD gene leads to the accumulation of citric acid, chelating a large amount of Fe³⁺ and disrupting the normal operation of the respiratory system in the bacteria which, in turn, affects biofilm formation. However, does Fe³⁺ affect biofilm formation through respiration in  B. cereus, and how does it mediate this effect? These answers are not yet known. Hopefully, our future experiments will be able to determine the answers to these questions.

Many studies have suggested that the level of ROS plays an important role in the regulation of spore differentiation initiation (Passalacqua et al. 2006), which was proven in our previous studies (Zhang et al. 2020). Therefore, we wanted to explore whether the function and activity of redox-related enzymes affect spore formation. The spore rate of the mutant ΔicaD was measured. Surprisingly, the spore yield of the mutant ΔicaD was different from that of  B. subtilis. Here, the sporulation rate of the mutant strain ΔicaD still reached approximately 60%, while Stage I sporulation was blocked after deletion of the icaD gene in  B. subtilis, and the sporulation rate was only 0.005% (Sheng et al. 1997). It has been reported that when the icaD gene is deleted from the TCA cycle in  S. aureus, the production of NADPH is hampered, which affects the redox balance in the cell, leading to impaired biofilm formation (Ilana et al. 2013). We hypothesized that the deletion of the icaD gene affected the intracellular redox balance, which could be the reason for the damage to spore formation in some stages. However, it has also been reported that the pH value of the culture affects spore formation (David and Sean 1995). We cannot rule out that the deletion of the icaD gene causes culture acidification that affects spore differentiation initiation. It has been reported that the spore formation process requires four stages, and the first major structural change in spore formation is misdivision (Liu et al. 2005). It remains to be determined which stages of spore formation in  B. cereus  0-9 were damaged when the icaD gene was deleted. In this work, we don't have the answer yet. But it's a fascinating question that may be answered in our future research.

**Conclusions**

**B. cereus 0-9** is a widely distributed and strongly adaptable potential biocontrol strain. We found that the deletion of icaD encoding IDH in the  B. cereus  0-9 genome severely affected the formation of biofilms. Fluorescence and CLSM assay results of the fusion reporter strains showed that the loss of the icaD gene also delayed the initiation time of  B. cereus  0-9 biofilm formation and changed the morphology of the biofilms. Our evidence indicated that the absence of IDH in the TCA cycle led to the accumulation of citric acid, which changed the acidification of the culture environment of the strain. Furthermore, the chelation activity of citric acid hindered the normal absorption of metal ions inside bacteria, resulting in reduced cell viability and affecting biofilm formation. The above results showed that IDH from the TCA cycle may regulate biofilm formation by regulating the intracellular redox balance. In addition, we found that when IDH was lost in  B. cereus  0-9, the sporulation rate was reduced, which was significantly different from
the stagnation of sporulation observed in *B. subtilis* due to interruption of the stage I sporulation process caused by *icdH* loss.

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