Deficiency of exopolysaccharides and O-antigen makes *Halomonas bluephagenesis* self-floculating and amenable to electrotransformation

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*Halomonas bluephagenesis*, a haloalkaliphilic bacterium and native polyhydroxybutyrate (PHB) producer, is a non-traditional bioproduction chassis for the next generation industrial biotechnology (NGIB). A single-sgRNA CRISPR/Cas9 genome editing tool is optimized using dual-sgRNA strategy to delete large DNA genomic fragments (>50 kb) with efficiency of 12.5% for *H. bluephagenesis*. The non-essential or redundant gene clusters of *H. bluephagenesis*, including those encoding flagella, exopolysaccharides (EPSs) and O-antigen, are sequentially deleted using this improved genome editing strategy. Totally, ~3% of the genome is reduced with its rapid growth and high PHB-production ability unaffected. The deletion of EPSs and O-antigen gene clusters shows two excellent properties from industrial perspective. Firstly, the EPSs and O-antigen deleted mutant rapidly self-floculates and precipitates within 20 min without centrifugation. Secondly, DNA transformation into the mutant using electroporation becomes feasible compared to the wild-type *H. bluephagenesis*. The genome-reduced *H. bluephagenesis* mutant reduces energy and carbon source requirement to synthesize PHB comparable to its wild type. The *H. bluephagenesis* chassis with a reduced genome serves as an improved version of a NGIB chassis for productions of polyhydroxyalkanoates (PHA) or other chemicals.
Next generation industrial biotechnology (NGIB) is a sustainable approach that aims at cost-effective production of various chemicals by overcoming the economic and technological challenges of current industrial biotechnology. NGIB employs robust microbial chassis possessing unique characteristics to synthesize bio-based products. Hence, the selection and engineering of promising microbial strains play a pivotal role in NGIB. Halomonas bluephagenesis, a rapidly growing haloalkaliphilic bacterium, has been developed as a low-cost and high yield chassis for NGIB to produce polyhydroxybutyrate (PHB) under open unstirred and continuous condition in seawater. Previous work has been conducted on metabolic engineering of H. bluephagenesis for production of poly(3-hydroxybutyrate) and its copolymers like poly(3-hydroxybutyrate-co-4-hydroxybutyrate), as well as chemicals, such as ecoinete, L-threone, and 3-hydroxypropionate. Moreover, cultivation of this halophile has also been successfully scaled up to the 5000-L pilot scale fermentor, which proved its feasibility as an industrial chassis for the production of various bioproducts.

In recent years, increasing research is focused on developing engineered H. bluephagenesis strains with a higher production efficacy via synthetic biology. Minimizing the genome size via deletion of nonessential genes can reduce the metabolic burden and impart more stability to the host cell. Genome reduction can generate high-performing microbial strains possessing industrially important physiological characteristics. Several genome-reduced strains of Escherichia coli, Bacillus sp., Corynebacterium glutamicum, and Streptomyces sp. have been developed to improve their metabolite production efficiency. Efficient genetic operating systems and genome editing tools are indispensable to accelerate the genome reduction in H. bluephagenesis. CRISPR/Cas9-based genome editing tool have been developed in H. bluephagenesis. However, it is less effective for editing large genomic sequences. Another requirement for designing high-performance engineered strain is transformation of large DNA fragments for heterologous metabolic pathway reconstruction. Till now, conjugation is the only available method for DNA transformation in H. bluephagenesis. This method allows the introduction of only plasmid DNA and not linear DNA fragments into the recipient cell. Moreover, DNA carrying capacity of plasmids is limited by their origin of replication. Therefore, introduction of large DNA fragment in H. bluephagenesis is not feasible by conjugation-based DNA transfer system. In this context, electroporation is a simple method that effectively transfers large-sized linear DNA fragments into host cell. Taken together, it is necessary to optimize the existing CRISPR/Cas9-based genome editing tool and enrich genetic transformation system to achieve a highly efficient genome-reduced strain of H. bluephagenesis that may serve as an excellent platform strain for NGIB.

Bacterial flagellum is a complex motor organelle composed of several proteins. Biosynthesis of flagella and its rotation are energy-intensive processes. In E. coli, flagellar biosynthesis accounts for almost 2% of the biosynthetic energy consumption by the cell. Interestingly, deletion of flagellar operon in Pseudomonas putida KT2440 improved ATP/ADP ratio by 30% and NADPH/NADP⁺ ratio by 20%. In addition, deletion of 76 genes relevant to flagella and pili formation increased polyhydroxalkanoates (PHA) content and its yield by 45.6 and 73.4%, respectively, in P. putida KT2440. The flagellar gene cluster in H. bluephagenesis is located upstream of a diguanylate cyclase gene. It is a 54.4 kb long fragment and does not contain any essential genes. Thus, it was assumed to be a suitable target to test the genome editing tool for large DNA fragment deletion in H. bluephagenesis. Moreover, it is expected that deletion of the flagellar gene cluster would reduce energy requirement of the cell by preventing flagellar biosynthesis.

Exopolysaccharides (EPSs) are extracellular, high-molecular-weight polymers that are crucial for biofilm formation. EPSs play essential roles in host-pathogen interactions and are believed to protect cells from environmental stress such as extreme pH, antibiotic or desiccation. EPSs also act as autoagglutinins, which mediate microbial self-flocculation. EPSs synthesis increases requirement of carbon source in the cell because the carbon supply is redistributed between EPSs biosynthetic pathway and other physiological processes. Knocking out the EPSs bio-synthesis cluster in Haloferax mediterrani improved PHA production by 20%. H. bluephagenesis exhibited viscous liquefaction phenotype when cultured on agar plate. This implied that H. bluephagenesis may secrete certain amounts of EPSs. It is plausible that the deletion of EPSs biosynthetic pathway would save carbon consumption for other physiological processes in this strain. Thus, the gene clusters involved in EPSs synthesis were selected for simplifying the genome of H. bluephagenesis.

This study aims to improve the properties of H. bluephagenesis via deletion of redundant synthesis pathways and make it more suitable as a chassis for NGIB.

Results

Optimization of CRISPR/Cas9-based genome editing system for large DNA fragment deletion. In a previous study, a CRISPR-Cas9-based gene editing system for H. bluephagenesis has been constructed. However, the editing efficiency for large DNA fragments is not high enough. Previously, a 2.3 kb long DNA fragment was deleted with an efficiency of 16.67%. Thus, a more effective genome editing tool is urgently needed for editing multiple genes and large DNA fragments. Type II CRISPR-Cas system, including Cas9, only induce double-strand break (DSB) at target site. It leaves a long gap that affects homology-directed repair for large fragments editing (Fig. 1a). In order to improve editing efficiency for large fragments, we introduced a double sgRNAs strategy with 1 kb homologous arms (Fig. 1b). To test the deletion efficiency, a 54.4 kb flagellar gene cluster containing 57 CDS (Coding sequence) was selected as the target (Fig. 1c and Supplementary Data 1). A 20 kb fragment was first edited to compare the efficiency between single and double sgRNAs strategies. Then, 20 to 50 kb fragments were edited with double sgRNAs to evaluate the efficiency of our developed strategy. As shown in Fig. 1c, the deletion efficiency of double sgRNAs (53.1%) was better than that of single sgRNA (31.2%). Besides, double sgRNAs caused mutation at a rate of 93.7%, whereas single sgRNA led to only 34.3% for editing 20 kb fragment. This ensured a higher probability of obtaining mutants using double sgRNAs when more complex fragments were knocked out. As expected, when double sgRNAs were used to delete 30, 40, and 50 kb fragments, the corresponding editing efficiencies were 25, 25, and 12.5%, respectively. The 50 kb of flagellar gene cluster was knocked out via our optimized CRISPR/Cas9-based genome editing tool, though deletion efficiency decreased with the increase in DNA fragment length.

Deletion of capsular polysaccharide synthesis clusters. The wild-type strain of H. bluephagenesis exhibited viscous liquefaction phenotype when cultured on LB60 plate (Fig. 2a). This implied that H. bluephagenesis synthesized and secreted large amounts of EPSs outside the cell. Genome-scale bioinformatics analysis of H. bluephagenesis predicted a ~90 kb long gene cluster related to polysaccharide synthesis and efflux, and a 16 kb putative chondroitin synthesis gene cluster. The relative position of gene clusters on the genome were shown in Fig. 2b. As the
The aid of centrifugation would greatly facilitate the recovery of cells from culture after fermentation. Furthermore, as shown in Fig. 3b, self-agglutination efficiency of ΔPS124 was influenced by the NaCl concentration. Self-agglutination was obviously low (~10%) when NaCl concentration was below 0.2 M. With gradual increase in NaCl concentration to 0.6 M, self-agglutination efficiency increased to almost 90% and became stable with further increase in NaCl concentration to 1 M. In summary, ΔPS124 exhibited the best self-agglutination efficiency above 0.6 M NaCl.

Exploring the self-agglutination mechanism of ΔPS124. Bacterial autotransporters, exopolysaccharides, flagellin protein, Type IV pilus, or extracellular DNA are parts of biofilm. These components act as autoagglutinins, which result in self-agglutination. However, in this study, knockout of EPSs clusters in H. bluephagenesis resulted in self-agglutination of cells.

Seeking possible agglutinins of ΔPS124. Transcriptome analysis was carried out to provide some suggestions about the underlying mechanism of self-agglutination at molecular biology level. Self-agglutination is generally mediated by surface proteins, such as autotransporters or Type IV pili20. Transcriptome analysis revealed that only 5 genes were significantly upregulated in the strain ΔPS124 (Supplementary Table 2). Among them, a filamentous hemagglutinin (FHaB), a 4-hydroxyphenylpyruvate dioxygenase (HppD), which was also annotated as hemolysin, and a hypothetical protein (Hyp) were initially speculated to be the key proteins related to self-agglutination. The remaining two upregulated genes encoded succinate-semialdehyde dehydrogenase (GabD) and gamma-aminobutyrate:alpha-ketoglutarate aminotransferase (DoeD), respectively. GabD, DoeD, and glutamate dehydrogenase (GdhA) participate in glutamate presence of essential genes shown in Supplementary Data 2, the 90-kb long gene cluster was divided into four segments by five predicted essential genes (Fig. 2c and Supplementary Table 1). Firstly, two segments, named as PS1 (16,861 bp) and PS2 (22,861 bp), respectively, were chosen for knockout to interrupt extracellular polysaccharide synthesis. Unfortunately, deletion of individual PS1 (strain ΔPS1) or PS2 (strain ΔPS2), or both (strain ΔPS12), did not result in the loss of viscous liquefaction phenotype. Subsequently, the candidate gene cluster of chondroitin synthesis, named as PS3 (16,379 bp), was deleted. However, deletion of PS3 (strain ΔPS3) still had no obvious effect on the phenotypic change. Finally, the PS4 segment, which encoded four transporters, O-antigen ligase, and UDP-phosphate galactose phosphotransferase, was deleted based on ΔPS12. The resultant mutant ΔPS124 exhibited a dry phenotype when cultured on the LB60 plate (Fig. 2a). Interestingly, the liquid culture of ΔPS124 showed a rapid cell flocculation characteristic. With the settling time, more and more cells flocculated at the bottom of tube. Within 5 min, cell flocculation was visible. In 30 min, the cells settled down to the bottom leaving the liquid completely clear. In the case of the wild-type strain, culture was homogenous with no signs of flocculation (Fig. 2d).

Effect of NaCl concentration on self-agglutination efficiency of ΔPS124. The time curve of self-agglutination efficiency in 60MMG medium containing 1 M NaCl was shown in Fig. 3a. The self-agglutination efficiency was achieved at ~95% in 5 min, and it increased to ~96% in 15 min and attained stabilization. In contrast, flocculation efficiency was below 10% in TD1.0 strain. The sedimentation of the ΔPS124 cells due to self-agglutination without the aid of centrifugation would greatly facilitate the recovery of cells from culture after fermentation. Furthermore, as shown in Fig. 3b, self-agglutination efficiency of ΔPS124 was influenced by the NaCl concentration. Self-agglutination was obviously low (~10%) when NaCl concentration was below 0.2 M. With gradual increase in NaCl concentration to 0.6 M, self-agglutination efficiency increased to almost 90% and became stable with further increase in NaCl concentration to 1 M. In summary, ΔPS124 exhibited the best self-agglutination efficiency above 0.6 M NaCl.

Fig. 1 Flagellar gene cluster deletion using single or double sgRNAs. a, b Design and comparison between single and double sgRNAs. The right-angle arrows and “T” symbols represent promoters and terminators, respectively. c Designed positions of homologous arms and sgRNAs for flagellar gene cluster deletion (left). Editing efficiency of deleting various flagellar gene cluster with single or double sgRNAs (right). 32 colonies in each group were verified by PCR. Homozygous genotype (purple) means the deletion of flagellar cluster in mutants which produce one PCR product with the size of 2.4 kb. Heterozygous genotype (yellow) represents the occurrence of both wild type and deletion of flagellar cluster in mutants which produce two PCR products with the size of 2.4 and 1.7 kb, respectively. The electrophoresis results of PCR products were shown in Supplementary Fig. 1. H1, the upstream homologous arm of flagellar gene cluster; H20 (H30, H40, and H50), the downstream homologous arm of flagellar gene cluster for the deletion of 20 kb (30, 40, and 50 kb) fragment.
metabolism. The individual deletion of fhaB, hppD, or hyp or simultaneous deletion of the 3 genes in ΔPS124 did not affect the self-agglutination (Supplementary Fig. 2).

Furthermore, transcriptome data showed that 81 genes were down-regulated in ΔPS124 (Supplementary Table 2). Most of them encoded membrane proteins. Among them, a nitrite reductase complex and a cytochrome d ubiquinol oxidase complex were significantly down-regulated. Both the enzymes are reported to function under anoxic conditions or oxygen-limited conditions. This suggests that the absence of EPSs may improve the oxygen permeability of cell membrane.

Visible change in the cell surface of ΔPS124. SEM analysis was subsequently conducted to observe the morphological differences between TD1.0 and ΔPS124. The cell surface of TD1.0 was rough and covered with wrinkles. On the contrary, the cell surface of ΔPS124 was smooth with some globular protuberances distributed on it (Fig. 4). The result indicated that the loss of EPSs exposed the cell surface of ΔPS124, which might be closely related with its self-agglutination. Simultaneously, TEM analysis was also performed to observe the changes in the outer membrane.

Improved cell surface hydrophobicity of ΔPS124. Enhanced cell surface hydrophobicity is one of the causes for self-agglutination. The hydrophobicity of cell surface was measured by a modified phase partitioning assay. As expected, ΔPS124 had higher cell surface hydrophobicity (46.1% as measured by a modified phase partitioning assay) compared to TD1.0 (11.3%) in LB60 medium (Fig. 5). Notably, the hydrophobicity of ΔPS124 increased to 81.3% in 1.0 M NaCl solution, whereas the hydrophobicity of TD1.0 decreased to minus 47.8%. Similar results have been reported in Halomonas elongata, but the reason is not addressed and needs to be further clarified.

Deficiency of both O-antigen and EPSs is the key factor for self-agglutination. Self-agglutination was triggered when the PS4 cluster was knocked out in H. bluephagenesis, indicating that PS4 was closely related with self-agglutination. In order to clarify the specific genes directly attributing to self-agglutination, PS4 was divided into 3 parts (Part1, Part2, and Part3) to be cloned into
pSEVA321 vector (Fig. 6a). Several attempts to clone Part2 failed, which might be due to the toxicity of DUF4258 to the host cell (E. coli). The two constructed plasmids pSEVA321-Part1 and pSEVA321-Part3 were individually transformed into ΔPS124. The complementation of Part1 had no effect on self-flocculation (Supplementary Fig. 2c), whereas the complementation of Part 3 restored the full suspension of ΔPS124 (Fig. 6b). Simultaneously, the complementation of Part 3 maintained the cells with a dry phenotype when cultured on LB60 solid medium (Fig. 6b). Part3 encodes an O-antigen ligase (waaL), a UDP-phosphate galactose phosphotransferase (rfbP), a lipopolysaccharide biosynthesis protein (wzz), a hypothetical protein, and 3 glycosyltransferases (Fig. 6a). Among them, WaaL is the key enzyme for ligating O-antigen chains with the outer core of the lipopolysaccharides (LPS) and Wzz determines the length of O-antigen28 (Fig. 6c). Both waaL and wzz serve as the boundary of Part3, implying that these 7 genes might constitute an O-antigen gene cluster. Subsequently, the O-antigen cluster was knockout in the wild type to test whether its absence is the only factor causing self-flocculation. The mutant ΔO-antigen did not show a self-flocculation property in LB60 liquid medium and still exhibited viscous liquefaction phenotype on LB60 solid medium (Fig. 6b). Therefore, the presence of EPSs hindered self-flocculation even though O-antigen was deficient in H. bluephagenesis. These results demonstrated that self-flocculation of H. bluephagenesis could be attributed to the increase of cell surface hydrophobicity resulted from the simultaneous deficiency of EPSs and O-antigen.

Deletion of PS3 gene cluster and 50 kb of flagellar cluster on the basis of ΔPS124. In order to further reduce redundant gene clusters, the PS3 gene cluster (16,379 bp) was knocked out on the basis of ΔPS124, which resulted in a ΔPS1234 mutant. Subsequently, the 50 kb DNA fragment of flagellar cluster was knocked out on the basis of ΔPS1234, which resulted in a final ΔPS1234Δ50k mutant. In total, 121 kb DNA sequence was deleted via our optimized CRISPR/Cas9-based genome editing method, which accounted to ~3% of its whole genome. Interestingly, deletion of both PS3 gene cluster and 50 kb flagellar cluster did not affect the self-flocculation phenotype. This further confirmed that neither EPSs nor flagella acted as autoagglutinins and mediated the self-flocculation of our mutant strain.

The growth curves of TD1.0, ΔPS124 and ΔPS1234Δ50k cultured in LB60 medium were shown in Fig. 7a. The three strains had a similar growth pattern. However, the OD600 of ΔPS124 and ΔPS1234Δ50k was lower than that of TD1.0 at stationary phase. The difference in OD values might be attributed to self-flocculation. The mutant ΔO-antigen did not show a self-flocculation property in LB60 liquid medium and still exhibited viscous liquefaction phenotype on LB60 solid medium (Fig. 6b). Therefore, the presence of EPSs hindered self-flocculation even though O-antigen was deficient in H. bluephagenesis. These results demonstrated that self-flocculation of H. bluephagenesis could be attributed to the increase of cell surface hydrophobicity resulted from the simultaneous deficiency of EPSs and O-antigen.
floculation or the cell surface change (Fig. 4) of ΔPS124 or ΔPS123Δ50k. Even though the culture was resuspended before measuring OD_{600}, the cells of ΔPS124 and ΔPS123Δ50k rapidly flocculated and led to decreased OD values. Carbon source consumption and PHB synthesis of each strain were further analyzed at 36 h of fermentation in 60 MMG medium. The dry cell weight of the three strains were almost similar (~13 g/L). Likewise, the PHB production level of TD1.0, ΔPS124 and ΔPS123Δ50k was also comparable (~9 g/L) (Fig. 7b). As shown in Fig. 7c, the total reducing sugar of ΔPS124 and ΔPS123Δ50k in fermentation broth and supernatant was 1070 and 740 mg/L, and 700 and 600 mg/L, respectively. In contrast, the respective values of total reducing sugar for TD1.0 strain was 620 and 400 mg/L, respectively. This result indicated that mutant strain ΔPS124 could produce a similar amount of PHB by consuming less carbon source compared to TD1.0.

ΔPS123Δ50k transformed using electroporation. Previously, conjugation was the only DNA transfer method for _H. bluephagenesis_14. Probably, EPSs secreted on the cell surface formed a barrier which limited the access of macromolecules. Therefore, it is conceivable that the absence of EPSs may promote the permeability of exogenous DNA into cells and thus increase the chances of electroporation-mediated gene transfer in _H. bluephagenesis._

Considering the fact that _H. bluephagenesis_ is a halophile and transformation medium with high osmolarity improved the electrotransformation efficiency of _Bacillus subtilis_29, four kinds of media with different osmotic pressure (see “Materials and methods”) were used to test the electrotransformation efficiency of _H. bluephagenesis_ strains. Besides, two pulse voltages (1800 and 1 M NaCl solution. LB60 medium contains 1 M NaCl. Data are shown from three biological replicates. Error bars are SD (n = 3). TD1.0, _H. bluephagenesis_ TD1.0; ΔPS124, _H. bluephagenesis_ TD1.0 with deletion of PS1, PS2 and PS4 gene clusters.

**Fig. 5** Cell surface hydrophobicity of ΔPS124 and TD1.0 in LB60 medium or 1 M NaCl solution.

**Fig. 6** Deficiency of O-antigen is the key factor of self-floculation. a O-antigen biosynthesis genes in the PS4 cluster. b Self-floculation of TD1.0, ΔPS124, ΔPS124/pSEVA321-Part3 and ΔO-antigen after standing for 30 min and their mucoid/dry phenotype. The strains were cultured in LB60 medium for 24 h and used for self-floculation test (left). Morphology of TD1.0, ΔPS124, ΔPS124/pSEVA321-Part3, and ΔO-antigen cultured on LB60 agar plate (right). TD1.0, _H. bluephagenesis_ TD1.0; ΔPS124, _H. bluephagenesis_ TD1.0 with deletion of PS1, PS2, and PS4 gene clusters; ΔPS124/pSEVA321-Part3, ΔPS124 complemented with Part3 of the PS4 cluster; ΔO-antigen, TD1.0 with deletion of the 7 genes in Part3. c Diagram of the structure of lipopolysaccharide (LPS) in _E. coli._ The action positions of WaaL and Wzz are depicted with arrows. GlcN, N-acetylgalactosamine; Kdo, 3-deoxy-D-manno-octulosonate; Hep, heptose; Glc, glucose; Gal, galactose; Oligosaccharide, oligosaccharide unit of O-antigen; P, phosphate group.
bluephagenesis could not be transformed by electroporation under all the six tested electrotransformation conditions. In contrast, ΔPS1234Δ50k could be electrotransformed under five electrotransformation conditions. Low osmotic electrotransformation media with 0.5 M sucrose favored electroporation of ΔPS1234Δ50k. The transformation was inhibited in high or moderate osmotic medium comprising of 1 M sucrose or 0.5 M sorbitol and 0.5 M trehalose or 0.5 M sorbitol, 0.5 M mannose and 0.5 M trehalose. The highest pulse voltage of 2.5 kV showed a better transformation efficiency. Best electroporation efficiency of 400 colony-forming unit (cfu)/µg DNA was achieved by using 0.5 M sucrose at 2.5 kV.

In order to test if the plasmid was stable in the transformants, we extracted the plasmids from 50 transformants of ΔPS1234Δ50k. As shown in Fig. 8c and Supplementary Fig. 4, the plasmid pSEVA341 was transformed into ΔPS1234Δ50k by electrotransformation method and could replicate stably in this strain. This characteristic endows the possibility to further genetically engineer H. bluephagenesis and improve its performance as an excellent cell factory.

Discussion
As summarized in Fig. 9, we optimized the CRISPR/Cas9 genome editing tool by using dual-sgRNA strategy for large-sized DNA fragment deletion in H. bluephagenesis. The gene clusters related to flagella, EPSs, and O-antigen biosynthesis in the strain were deleted using the optimized strategy, leading to a genome reduction by ~3%. Surprisingly, the resulting mutant showed two excellent characteristics. Firstly, the deletion of EPSs and O-antigen conferred self-flocculation characteristics to the strain without affecting growth and PHB production. Secondly, DNA transformation into the EPSs and O-antigen deficiency mutant could be mediated by electroporation, which otherwise was not feasible in the wild-type strain.

Minimizing the genome without affecting cell growth and unique robustness is still a challenge. Many species including E. coli, and Bacillus sp., have undergone an intensive genome reduction. In several cases, genome reduction has boosted the performance of the microbial strain by influencing their physiological traits and improving their metabolite producing ability. In E. coli, production of L-threonine in a genome-reduced strain was 83% higher than that in wild-type strain. Likewise, a genome-reduced strain of Bacillus amyloliquefaciens lacking ~4.18% of the genome showed 10.4-fold increase in surfactin biosynthesis compared to the parent strain. Interestingly, deletion of 7.7% of the genome of Pseudomonas mendocina NK-01 improved the ATP/ADP ratio of the cell by 11 times and enhanced MCL-PHA accumulation by 114.8%.

Based on these previous studies, genome reduction was attempted for the first time in H. bluephagenesis by using dual-sgRNA CRISPR/Cas9 genome editing tool. We knocked out the non-essential gene clusters associated with the production of EPSs and the formation of flagella in H. bluephagenesis. The genome of H. bluephagenesis was reduced by sequentially deleting flagella, EPSs, and O-antigen gene clusters, resulting into ~3% genome reduction and the obtainment of an improved chassis. Strikingly, the mutant strain gained the self-flocculation ability and electroporation feasibility, the latter of which is a beneficial characteristic in terms of industrial application.

NaCl concentration influenced the self-flocculation property of H. bluephagenesis ΔPS124. This can be explained by the Derajan, Landau, Verwey, and Overbeek (DLVO) theory, which is a classical theory of colloidal stability. Bacteria usually exhibit a negative cell surface charge, which attracts the surrounding cations to form a double layer according to the DLVO theory.
The size of the double layer decreases with the increase of ionic strength. When the double layer decreases, the repulsion between cells also decreases, thus accelerating cell aggregation. When the NaCl concentration increased to 0.6 M, self-occulation led to faster sedimentation of cells as 90% of the cells settled down within 30 min (Fig. 3). Thus, it was possible to control the self-occulation of the strain by adjusting salt concentration. Self-occulation is very convenient for the recovery of fermentation products; therefore, it simplifies downstream processing during industrial production. Self-occulting cells can be separated from the fermentation broth rapidly without centrifugation or membrane filtration. This saves the capital and operating costs and energy consumption. Moreover, after harvesting the cells, supernatant can be reused for repeated fermentation, and thus reducing generation of wastewater and effluent treatment cost34. In Halomonas campaniensis LS21, deletion of the electron transferring flavoprotein operon led to self-occultation of the cells34. The self-occulting H. campaniensis LS21 was cultured in a wastewaterless, open, unsterile, and continuous fermentation system for four runs for PHA production. However, the PHA production in the mutant was lower compared to its wild type34. In our case, the growth and PHB production of self-occulting mutants of H. bluephagenesis (ΔPS124 and ΔPS1234Δ50k) were not impaired, although the loss of EPSs can be seen directly. Without synthesis of EPSs, cells may have saved a lot of carbon sources. Consistently, the total reducing sugar concentration in the fermentation broth for ΔPS124 and ΔPS1234Δ50k was higher than that of the wild type. Therefore, H. bluephagenesis ΔPS124 and ΔPS1234Δ50k could act as candidate chassis for the production of PHA and even other valuable bio-products.

Fig. 8 Electrotransformation of TD1.0 and ΔPS1234Δ50k. a, b Cells were electrotransformed with 500 ng of pSEVA341. The LB60 agar plates (25 μg/mL of chloramphenicol) were incubated at 37 °C for 48 h. c Agarose gel electrophoresis of the plasmid extracted from TD1.0, ΔPS1234Δ50k, and electrotransformed ΔPS1234Δ50k. The black arrows show the bands of the native plasmid from H. bluephagenesis and pSEVA341, respectively. TD1.0, H. bluephagenesis ΔTD1.0; ΔPS1234Δ50k, H. bluephagenesis TD1.0 with deletion of PS1, PS2, PS4, and PS3 gene clusters and flagellar gene cluster.

Table 1 Electrotransformation efficiency of TD1.0 and ΔPS1234Δ50k under different electroporation media and voltages.

| Strain       | Voltage (kV) | 1.0 M sorbitol 0.5 M trehalose | 0.5 M sorbitol 0.5 M mannose 0.5 M trehalose | 1.0 M sucrose 0.5 M sucrose |
|--------------|--------------|-------------------------------|---------------------------------------------|-----------------------------|
| TD1.0        | 2.5          | 0                             | 0                                           | 0                           |
|              | 1.8          | -                             | -                                           | 0                           |
| ΔPS1234Δ50k  | 2.5          | 2.7 ± 4.6                     | 1.3 ± 1.2                                   | 2.0 ± 2.0                   |
|              | 1.8          | -                             | -                                           | 20.7 ± 1.2                  |

<: The voltage was not considered. Three parallels were set for each group.

Fig. 9 Illustration of the deletion of EPSs and O-antigen synthesis gene clusters. a EPSs and O-antigen synthesis gene clusters of H. bluephagenesis TD1.0 were deleted with dual-sgRNA CRISPR/Cas strategy, and resulted in H. bluephagenesis ΔPS124. b H. bluephagenesis ΔPS124 can self-occult in the range of 0.4-1.0 M NaCl concentration, while H. bluephagenesis ΔPS124 cannot. c H. bluephagenesis ΔPS124 can be transformed by electroporation, whereas H. bluephagenesis ΔPS124 cannot.
genes were not included as our targets for further genetic verification due to their low transcription level. Another possible autoagglutinin reported to cause self-flocculation is flagellar protein98. However, deletion of flagella gene cluster in H. bluephagenesis ΔPS124 had no visible effect on self-flocculation, indicating flagella is not the contributing factor to self-flocculation of ΔPS124. The lack of O-antigen has been reported to enhance the cell surface hydrophobicity of gram-negative bacteria.37 Similarly, we found that the self-flocculation ΔPS124 showed increased surface hydrophobicity due to the O-antigen deficiency. An increase in cell surface hydrophobicity is a physical factor for self-flocculation. However, the O-antigen mutant did not have the self-flocculation ability when the synthesis of EPSs was not disrupted. Our result indicates that EPSs might function as a shield to protect cells from self-flocculation of H. bluephagenesis. Similar function of capsular polysaccharides has been reported that it can block the function of the self-recognizing protein antigens98, which is the key protein causing self-flocculation in E. coli, through physical shielding.38 EPSs are usually one of the causes of self-flocculation20,39,40, which is not the case for H. bluephagenesis. The disruption of O-antigen synthesis could lead to self-flocculation. The wzm ABC O-antigen transporter gene knockout mutant of Synchococcus elongatus was found to be capable of self-flocculation41. Collectively, the deficiency of EPSs and O-antigen is indispensable for self-flocculation of ΔPS124.

It is speculated that deletion of surface EPSs and O-antigen may enhance the permeability of the cell membrane and facilitate the movement of some substances into and out of the cells. So, we employed the NPN (1-N-phenylglycylphthalamine) method to test the outer membrane permeability of our mutant strains. This method was used in many studies for outer membrane modification82,43. However, no difference was observed between wild type and ΔPS1234Δ50k (Supplementary Fig. 3e). This indicated that NPN might not be excluded by the EPSs or O-antigen of H. bluephagenesis. We further tested whether macromolecular plasmid DNA could be transformed into ΔPS1234Δ50k by electroporation. EPSs and O-antigen may be one of the reasons that prevented DNA from entering cells. Surprisingly, ΔPS1234Δ50k could be transformed using electroporation method. The electrotransformation method allows the introduction of linear DNA fragments and is urgently needed for high-throughput gene editing and mutant library screening in H. bluephagenesis. Using linear DNA fragments for transformation will save time and cost of plasmid construction. Therefore, cells that can be electrotransformed are the basis for large-scale genetic manipulation in the future. There are various methods of improving electrotransformation efficacy, ranging from physical parameter optimization to genetic manipulation. In Dietzia sp., optimization of the physical parameters including electric field strength, electroporation time, and chemical concentration obviously improved electrotransformation efficacy44. In addition, a thinner cell wall and deletion of the genes involved in peptidoglycan synthesis increased electrotransformation efficacy of Corynebacterium glutamicum45,46. Deletion of EPSs and O-antigen gene clusters realizing electrotransformation of H. bluephagenesis is a first report and a further modification to obtain a high-efficient electrotransformation strain is making progress.

In conclusion, CRISPR/Cas9 genome editing tool has been optimized by using dual-sgRNA to delete the redundant synthesis pathways of flagella, EPSs and O-antigen in H. bluephagenesis, and a self-flocculating mutant with less carbon and energy consumption was obtained. The mutant was able be accumulate similar amounts of PHB compared to its wild type. Notably, it is a breakthrough that deletion of EPSs and O-antigen gene clusters realizes electrotransformation of H. bluephagenesis. Taken together, our work provides a non-traditional bioproduction chassis with great potential for NGIB to overcome economic and technological challenges of current industrial biotechnology.

### Methods

**Strains, plasmids, and culture conditions.** All strains and plasmids used in this study are shown in Supplementary Table 3. H. bluephagenesis TD1.0 was selected as the original chassis strain in this study47. Escherichia coli DH5α and S17-1 were used for plasmids construction and conjugation. H. bluephagenesis TD1.0 and its derived strains were cultured in LB60 medium (10 g/L tryptone, 5 g/L yeast extract, and 60 g/L NaCl)93. E. coli was cultured in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl). The compositions of the 60MMG medium are: 60 g/L NaCl, 30 g/L glucose, 1 g/L yeast extract, 2 g/L NH4CL, 0.2 g/L MgSO4, 9.65 g/L NaHPO4·12H2O, 1.5 g/L KH2PO4, 10 mL/trace element solution I and 1 mL/trace element solution II. The compositions of trace element solution I are: 100 mg/L ZnSO4·7H2O, 30 mg/L MnCl2·4H2O, 300 mg/L H3BO3, 200 mg/L CoCl2·6H2O, 10 mg/L CuSO4·5H2O, 20 mg/L NiCl2·6H2O, 30 mg/L Na2MoO4·2H2O. The pH of 60 MMG was adjusted to approximately 8.5 using 5 M NaOH94. The final concentration of 25 μg/ mL kanamycin, 25 μg/ mL chloramphenicol, or 100 μg/ mL spectinomycin was added as needed.

**Construction of plasmids.** Plasmids carrying CRISPR array and homologous arms (1000 bp each) were constructed by Gibson Assembly. The plasmid backbone was amplified using the primers pSEVA241F and pSEVA241R from pSEVA24134. Homologous arms were cloned from the genomic DNA of H. bluephagenesis by PCR. For the design of sgRNA spacer, a 20 bp of protospacer with ~50% GC content before NGG (PAM) was manually chosen using Snapgene. The CRISPR array for expressing sgRNAs were synthesized by GENEWIZ (Suzhou, China). The diagram for the construction of plasmids carrying CRISPR arrays and homologous arms is shown in Supplementary Fig. 5. To clone 3 parts of PS4, each part was amplified by PCR and then recombined into pSEVA321 by Gibson Assembly. The sequences of the primers and synthetic DNA are listed in Supplementary Table 4 and Supplementary Table 5.

**Conjugation.** E. coli S17-1 containing the target plasmid as the donor strain was cultivated in LB medium supplemented with appropriate antibiotic. H. bluephagenesis, the recipient strain, was cultured in LB60 medium supplemented with appropriate antibiotics. The overnight cultures of both the strains were inoculated (1% v/v) into fresh LB and LB60 medium supplemented with appropriate antibiotics for 6 h, respectively. A total of 1 mL of each culture was harvested by centrifugation (6000 x g, 2 min), washed, and resuspended in 50 μL of LB or LB60 medium. The resuspended cells were mixed and dripped on LB20 agar plate. After overnight incubation at 37 °C, the moss was scraped up and resuspended in 100 μL of LB60 medium. Finally, the resuspended cells were spread on LB60 plate supplemented with appropriate antibiotics and incubated at 37 °C for 24–96 h to obtain colonies.

**Genome editing.** Genome editing was carried out based on a double-plasmid system14. Firstly, plasmid pQ08, which was designed to express Cas9 protein, was transformed into H. bluephagenesis by conjugation as described in 4.3. The derived plasmid pPO241 carrying homologous arms and CRISPR array were transformed into H. bluephagenesis/pQ08 by conjugation. Recombinant colonies were screened on LB60 medium with 25 μg/mL chloramphenicol and 100 μg/mL spectinomycin. The colonies were randomly selected and identified by colony PCR using the primers shown in Supplementary Table 4.

**Determination of self-flocculation efficiency.** H. bluephagenesis strains cultured in LB60 medium for 24 h were used directly to measure the time curve of self-flocculation efficiency. ΔPS124 grown in 60MMG medium for 48 h was harvested, and then resuspended in the same volume of different concentrations of NaCl solution ranging from 0 to 1.0 M to measure the effect of salt concentration on self-flocculation efficiency.

For measurements, 15 mL culture was put into a 15 mL-centrifuge tubes, shaken thoroughly and then allowed to stand 0–30 min. A total of 200 μL liquid was collected at 0.5 cm away from the liquid surface and then its OD600 was tested by Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, USA). For each experimental group, three parallel samples were set. The self-flocculation efficiency of each sample was calculated according to following equation:

\[
\text{Flocculation efficiency(\%) = (1 - b/a) x 100\%}
\]

where a is the OD600 before rest and b is the OD600 after rest34.

**Shake-flask culture of H. Bluephagenesis.** Single colonies were cultured in LB60 medium overnight. Then the cultures were inoculated (1% v/v) into 50 mL fresh LB60 medium, and subcultured for 10 h to serve as seed cultures. The seed cultures

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were inoculated (5% v/v) into 50 mL 60MMG medium containing 30 g/L glucose in a 500 mL shake flask. Shake flasks were incubated under 200 rpm at 37 °C for 24 h. 10 mL of electroporation medium was transferred by centrifugation at 5000 × g for 10 min and then washed three times with electroporation medium. Four kinds of electroporation media with different osmolality were used (0.5 M sucrose with lower osmolality; 1.0 M sucrose with moderate osmolality; a mixture of 0.5 M sorbitol and 0.5 M trehalose with high osmolality; a mixture of 0.5 M sorbitol, 0.5 M mannose and 0.5 M trehalose with high osmolality). Each 50 mL of original culture was suspended in 1 mL of pSEVA341 plasmid (100 ng/mL) and then transferred to an electroporation cuvette (0.2 cm gap, Bio-Rad). After incubation for 5 min, the cuvettes were exposed to a single electrical pulse using a Gene-Pulser II (Bio-Rad) set at 25 μF, and 200 Ω. The above operations were carried out at 4 °C. Immediately following the electroporation, 1 mL of SOC60 recovery medium (20 mM glucose, 20 g/L tryptone, 5 g/L yeast extract, 60 g/L NaCl, 2.5 mM KC1, 5 mM MgCl2, and 5 mM MgSO4, pH 7.5) was added into the cuvette, mixed, and transferred to a 1.5 mL centrifuge tube. After incubation at 37 °C and 200 rpm for 4–5 h, the cells were plated on LB60-agar plates containing 25 μg/mL chloramphenicol for selection of transformed cells. Finally, the plates were incubated at 37 °C for 48 h, and the transformants were enumerated. A total of 50 transformants were randomly picked up and cultured overnight in LB60 liquid medium supplemented with chloramphenicol (25 μg/mL), and then the plasmids were extracted with Plasmid Extraction Kit and verified by agarose gel electrophoresis.

Assays of total sugar concentration. The total sugar concentration was determined using the anthrone reagent48. 1 mL of anthrone reagent was added into 1 mL of sample, and the mixture was heated in boiling water for 10 min. Then, OD490 was measured using a microscope reader. Calibration curves were constructed with 0–100 mg/mL glucose.

Scanning electron microscopy (SEM). For SEM, cells cultivated in LB60 medium for 24 h were harvested by centrifugation at 3000 × g for 3 min, and then washed with PBS buffer (pH 7.2). Subsequently, the cells were fixed with 1 mL of 2% glutaraldehyde dissolved in PBS buffer (pH 7.2) and kept at room temperature for 30 min and then at 4 °C overnight. Then, the cells were washed with PBS buffer three times. After that, fixed cells were dehydrated by using different gradients (v/v) of ethanol solutions, 50, 70, 80, 90, and 100%. Samples were vacuum dried and coated with Au before SEM visualization (SU8010, Hitachi, Japan).

Hydrophobicity assay. The hydrophobicity of cell surface was determined by modified phase partitioning assay based on microbial adhesion to hydrocarbon (MATAD) test. The tested strains were cultivated for 24 h in LB60 medium. The cultures were centrifuged and resuspended in 1 M NaCl solution. A total of 5 mL suspension or cell solution was dispensed into a 15 mL tube. After a brief shake, 1 mL of the cell suspension or culture was taken to obtain the initial OD600. Then, 1 mL of xylene was added into the residual sample in 15 mL tube. The tubes were vortexed thoroughly for 1 min. After a complete phase separation, the lower aqueous phase was taken to obtain the ultimate OD600. The cell surface hydrophobicity was calculated as (initial OD600–ultimate OD600)/initial OD600 × 100%.27 For each group, three replicates were set.

Transcriptome analysis. H. bluephagenesis TD1.0 and DPS124 were cultured in 60MMG medium under 200 rpm at 37 °C for 24 h. Cells were harvested by centrifugation at 10,000 × g for 2 min and stored in a refrigerator at −80 °C before sequencing. Each group has two replicates. RNA extraction, transcriptome sequencing, data analysis, and differential expression analysis were performed by Novogene Co., Ltd. (Beijing, China)49. Briefly, total RNA extraction was performed by using TRIzol reagent (TIANGEN Biotech. (Beijing) Co., Ltd.) according to the manufacturer’s instructions. mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Sequencing was performed on an Illumina Novaseq (Bio-Rad) set at 25 μF, 100 Ω, and 200 °C, respectively. The temperature of the column was set at 80 °C and was maintained for 5 min, the cuvettes were exposed to a single electrical pulse using a Gene-Pulser II (Bio-Rad) set at 25 μF, and 200 Ω. The above operations were carried out at 4 °C. Immediately following the electroporation, 1 mL of SOC60 recovery medium (20 mM glucose, 20 g/L tryptone, 5 g/L yeast extract, 60 g/L NaCl, 2.5 mM KC1, 5 mM MgCl2, and 5 mM MgSO4, pH 7.5) was added into the cuvette, mixed, and transferred to a 1.5 mL centrifuge tube. After incubation at 37 °C and 200 rpm for 4–5 h, the cells were plated on LB60-agar plates containing 25 μg/mL chloramphenicol for selection of transformed cells. Finally, the plates were incubated at 37 °C for 48 h, and the transformants were enumerated. A total of 50 transformants were randomly picked up and cultured overnight in LB60 liquid medium supplemented with chloramphenicol (25 μg/mL), and then the plasmids were extracted with Plasmid Extraction Kit and verified by agarose gel electrophoresis.

Statistics and reproducibility. Data was analyzed with GraphPad Prism software and shown as means ± standard deviation (SD). Statistical significance was performed using unpaired t test at three significance levels (*P < 0.05).

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
The RNA-seq data was deposited in the National Center for Biotechnology Information database (accession number PRJNA721089). All other data are available from the corresponding author on reasonable request. Source data underlying all figures have been provided as Supplementary Data 3.

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Author contributions
T.X.: methodology, investigation, formal analysis, data curation, and writing - original draft. J.C.: Data curation and writing - original draft. R.M.: Data curation and writing - review & editing. L.L. and Z.X.: Methodology, G.-Q.C.: Resources and writing-review & editing. H.X. and J.H.: Methodology, supervision, and writing- review & editing.

Competing interests
The authors declare no competing interests.

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