Rational flux-tuning of Halomonas bluephagenesis for co-production of bioplastic PHB and ectoine

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Ectoine, a compatible solute synthesized by many halophiles for hypersalinity resistance, has been successfully produced by metabolically engineered Halomonas bluephagenesis, which is a bioplastic poly(3-hydroxybutyrate) producer allowing open unsterile and continuous conditions. Here we report a de novo synthesis pathway for ectoine constructed into the chromosome of H. bluephagenesis utilizing two inducible systems, which serve to fine-tune the transcription levels of three clusters related to ectoine synthesis, including ectABC, lysC and asd based on a GFP-mediated transcriptional tuning approach. Combined with bypasses deletion, the resulting recombinant H. bluephagenesis TD-ADEL-58 is able to produce 28 g L⁻¹ ectoine during a 28 h fed-batch growth process. Co-production of ectoine and PHB is achieved to 8 g L⁻¹ ectoine and 32 g L⁻¹ dry cell mass containing 75% PHB after a 44 h growth. H. bluephagenesis demonstrates to be a suitable co-production chassis for polyhydroxyalkanoates and non-polymer chemicals such as ectoine.
Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is one of the best well-known compatible solutes produced by halophiles for hypersalinity resistance. Ectoine was reported to show protections for living cells by enhancing the stabilities of proteins, nucleic acids, and cell membranes, enabling survival of halophiles under the circumstances of hypersalinity, high temperatures, extreme pH and exposure to chemical agents2–7. Therefore, ectoine has been exploited for commercial applications as an ingredient for cosmetics8, medicines9, and organ transplantation maintenance10. However, the high production cost is still unacceptable for wider applications11.

Commercially, fermentative production of ectoine by microorganisms under sterile bioprocesses is the major solution to meet its increasing demands12. The major biosynthesis pathway of ectoine containing its operon ectABC encoding l-2,4-diaminobutyrate acetyltransferase (ectA), l-2,4-diaminobutyrate transaminase (ectB) and ectoine synthase (ectC), respectively, has been clearly characterized to be a highly conservative cluster across halophile species, allowing three-step bio-activation to form ectoine from l-aspartate–β-semialdehyde (ASA), an important intermediate involved in several amino acids synthesis, such as threonine and lysine13–15. Interestingly, many halophiles also contain ectoine hydrolase encoded by ectD for converting ectoine into 5-hydroxyectoine as both carbon- and nitrogen-source used under nutrient-limited conditions, which is a dominating bypass limiting the high-level intracellular accumulation of ectoine16,17.

Recent progresses have exemplified the enhanced production of ectoine by recombinant halophiles, halophilic archaea, E. coli and Corynebacterium glutamicum employing various synthetic biology strategies. Specifically, halophiles were first grown in media with a high salt concentration for sufficient ectoine synthesis, subsequently cells were quickly transferred into desalted water for effective secretion of ectoine into extracellular space, termed ‘bacterial milking’18,19. Interestingly, it was reported that the deletion of TeaABC transporter in Halomonas elongata DSM 2581 led to an efficient secretion of ectoine in extracellular space during a high-cell-density fermentation20,21. Additionally, the heterologous expression of ectABC cluster from halophiles by engineered model microorganisms enabling sufficient ectoine biosynthesis has been achieved: ectABC from Chromohalobacter salsexigens and Halomonas elongata DSM 2581, the most studied cluster, were overexpressed in E. coli for enhanced production of ectoine from l-aspartate and glucose24,22. Remarkably, metabolically engineered E. coli W3110 and C. glutamicum with bypass deletion, transcriptional (promoter) and translational (RBS) tuning based on high-throughput combinatorial constructs and bioprocess optimizations, were successfully used to direct metabolic fluxes towards ectoine synthesis from glucose for efficient ectoine synthesis6,23.

Compared with current industrial biotechnology (CIB) based on model microorganisms, next generation industrial biotechnology (NGIB) based on Halomonas bluephaugenesis TD01, a natural poly(3-hydroxybutyrate) (PHB) producer, has been successfully developed for achieving cost-effective production under open unsterile and continuous growth conditions. PHB, a family member of microbial biopolymesters synthesized by many bacteria and archaea including E. coli, Pseudomonas putida and Cupriavidus necator et al., is a model biodegradable polymer for commercial applications28–32. H. bluephaugenesis has been successfully engineered to produce cost-effective PHB33–35 from lab-scale to pilot-scale (5000-L) fermentation. It has become highly desirable to engineer H. bluephaugenesis as a versatile chassis for productions of valuable chemicals at a competitive cost, or for co-production of PHA (polyhydroxyalkanoates, or PHB) with a small molecular chemical to increase the process economy36,37. H. bluephaugenesis could be a suitable host to engineer for ectoine production due to its fast growth and high PHB accumulation under high-salt conditions. So far H. bluephaugenesis has been manipulated via various approaches including CRISPR/Cas934, stimulus response-based flux-tuning (SR-FT)35, genomic integrations, inducible promoter mining15, and promoter engineering39. These concreted efforts enable rational strain engineering of complexity for synthesizing diverse metabolic targets, strongly expanding the capability of H. bluephaugenesis as a versatile production chassis. Generally, static optimization is a widely used strategy to fine-tune the pathways involved in multi-gene regulation largely dependent on high throughput constructs and machine learning-based analysis40–42, leading to high cost of clone construction with a long R&D cycle.

This study aims to chromosomally engineer H. bluephaugenesis for enhanced production of ectoine from glucose using “stimulus response-based flux-tuning” method (Fig. 1). Specifically, the ectoine titer reaches 6.3 and 28 g L−1 during a 48 h growth in 500 mL shake flask and 28 h fed-batch cultivation in 7-L bioreactor, respectively, by chromosomally engineered H. bluephaugenesis TD-ADEL-38 hide-tuning fine-tuned ectoine synthesis pathway. At the same time, co-production of ectoine and PHB is also studied for possible economic improvement43–45, which results in 8 g L−1 ectoine and 32 g L−1 dry cell mass (DCM) containing 75% PHB after 44 h fed-batch fermentation.

Results

Identification of ectoine synthesis pathway. Previous studies reported that intracellular accumulation of ectoine plays the main role in hypersalinity resistance in most halophilic bacteria46, of which H. bluephaugenesis is the one engineered as the chassis for NGIB47. As a metabolic target and compatible solute, cell growth characterization was performed to study the impact between ectoine and NaCl in the presence of various ectoine and NaCl concentrations, respectively (Fig. 2a). Interestingly, ectoine provides tolerance to H. bluephaugenesis grown under high- and low-salt conditions, and minor effects were observed in a 60MMG medium regardless of ectoine concentrations (Fig. 2a and Supplementary Fig. 1). It is important to note that the de novo synthesis pathway of ectoine has been identified, it is highly conservative across species (Fig. 1). Based on the genomic analysis of H. bluephaugenesis, the ectABC gene cluster encoding the major enzymes for ectoine synthesis (Fig. 1) was annotated by several databases including RAST and KEGG. Particularly, the nucleotide blast results from NCBI database show that genes ectA, ectB, and ectC of H. bluephaugenesis shared 86.46%, 86.05%, and 80.30% identity, respectively, compared with the ones from H. elongata DSM2581. Two strategies were employed to verify the functionality of ectABC cluster: (I) prototyping ectoine production by the start host, H. bluephaugenesis TD1.0; (II) heterologous over-expressing ectABC for ectoine production by recombinant E. coli22.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was applied to assay the accumulation of ectoine by H. bluephaugenesis TD1.0 cultivated in a 60MM medium supplemented with 30 g L−1 glucose in shake flask studies. Results showed that the biosynthesis ectoine shared the same retention time of mass spectra with ectoine standard, which is consistent with the secondary analysis results of LC-MS/MS (Supplementary Fig. 2). Furthermore, high performance liquid chromatography (HPLC) was used to quantify the initial production of ectoine by H. bluephaugenesis TD1.0 after cell lysis, as most halophiles accumulate ectoine intracellularly for resisting high salt circumstances (Supplementary Fig. 3). As a result, a production titer of 0.63 g L−1 ectoine was obtained from 9 g L−1 DCM containing...
62 wt% PHB (Fig. 2b), confirming the function of ectABC cluster in H. bluephagenesis. Secondly, ectABC from H. bluephagenesis was cloned into the medium-copy-number plasmid pSEVA321, for heterogenous expression by recombinant E. coli DH5α under the control of P_{lac} promoter induced by IPTG (Fig. 2c). The titer of ectoine varied from 2 to 4 g L\(^{-1}\) in the presence of different IPTG concentrations for 48 h cultivation in the MME medium (Fig. 2d), further demonstrating the function of ectABC in H. bluephagenesis. Thus, more efforts would be conducted to improve the production yield of ectoine by employing various metabolic engineering approaches (Fig. 1).

Tuning the ectABC expression for enhanced ectoine synthesis. H. bluephagenesis was generally engineered for diverse PHA productions requiring a nitrogen limited medium, or a 60MM medium supplemented with 30 g L\(^{-1}\) glucose for shake flask cultivations. As ectoine is an aspartate derived compound heavily depending on both carbon- and nitrogen-catabolism, it is necessary to reinforce the nitrogen supply via a medium modification. Even more important, transcriptional tuning of ectABC cluster via adding L-aspartate as precursor is significant for channeling more fluxes to ectoine, while avoiding the possibility of endogenous negative feedback control on the ectABC expression.

Based on the above understanding, a T7-like induced system was constructed to overexpress ectABC on a plasmid-based system in recombinant H. bluephagenesis TD1.0 in the presence of various concentrations of IPTG (Fig. 3a)\(^{35}\). Specifically, 100 mM aspartate was added in the cultural medium, namely MME (see methods), to ensure the metabolic fluxes flowing towards ectoine synthesis. As a result, the optimal transcriptional level for ectABC expression was determined after the induction of 20 mg L\(^{-1}\) IPTG, the maximum production titer of ectoine reached 2.38 g L\(^{-1}\) (Fig. 3b), indicating that a medium optimization should be conducted in the presence of 20 mg L\(^{-1}\) IPTG in shake flask studies. Therefore, factorial analysis was performed to search the optimized concentration of NaCl in the MME media for enhanced ectoine production by recombinant H. bluephagenesis harboring ectABC expression module induced by 20 mg L\(^{-1}\) IPTG. The 60MME medium containing 60 g L\(^{-1}\) NaCl demonstrated the highest 2.82 g L\(^{-1}\) ectoine when cell growth and ectoine synthesis was balanced (Fig. 3c). The ectoine titer was comparable at a higher salt concentration, but the cell growth was inhibited. In contrast, a low NaCl concentration also significantly decreases the production titer of ectoine (Fig. 3c).

However, the use of aspartate as a nitrogen source is not suitable for cost-effective industrial ectoine production due to its high cost. Therefore, urea, a low-cost nitrogen source replaced aspartate for de novo synthesis of ectoine (Fig. 1). On the basis of ectABC flux-tuning and NaCl concentration modification, a urea optimized concentration was found to be at 3 g L\(^{-1}\), and was added to a 60MMG medium, termed 60 MU (see methods). This resulted in a 2.83 g L\(^{-1}\) ectoine produced by recombinant H. bluephagenesis harboring ectABC expression module induced by 20 mg L\(^{-1}\) IPTG in shake flasks (Fig. 3d), which is close to that cultured in 60MME medium. Addition of urea had a negligible impact on cell growth, yet significantly decrease the PHB content when urea exceeded 1 g L\(^{-1}\). This info is an important hint for co-production of PHB and ectoine in this study.

Deletion on degradation genes for enhanced ectoine synthesis. Ectoine can be degraded in two major pathways encoded by doeA (EC 3.5.4.44) and ectD (EC 1.14.11.55) (Fig. 1)\(^{48}\), forming N-α-acetyl-L-2,4-diaminobutyrate (N-α-ADABA) and 5-hydroxyxycetaine, respectively. Both of these two genes were found in the genome of H. bluephagenesis via genomic analysis, they function...
to reduce ectoine accumulation. It is thus necessary to eliminate these two branches for blocking the degradation of ectoine by deleting doeA and ectD (Fig. 1). A CRISPR/Cas9 mediated gene editing tool was employed to generate single-knockout H. bluephagenesis TD-A (ΔdoeA), and double-knockout H. bluephagenesis TD-AD (ΔdoeA, ΔectD), respectively (Fig. 4a).

Deletion of doeA and ectD were confirmed based on colony PCR using two primer designs (Fig. 4a). Sequences of sgRNA designed for gene deletions were listed in Supplementary Table 2, respectively.

Subsequently, ectoine production by H. bluephagenesis TD01 derived strains H. bluephagenesis TD1.0, H. bluephagenesis TD-A and H. bluephagenesis TD-AD harboring ectABC expression module, respectively, induced using 20 mg L⁻¹ IPTG were conducted in shake flasks. Recombinant cells were cultured in 60MME (Fig. 4b) and 60MMU medium (Fig. 4c), respectively. Results showed that doeA deletion led to a 35% increase on ectoine titer, reaching up to 3.1 g L⁻¹, and further deletion on ectD increased ectoine to only 3.2 g L⁻¹ (Fig. 4c) when cultured in the 60MMU medium. Interestingly, only 5% and 15% increases were observed by H. bluephagenesis TD-A and H. bluephagenesis TD-AD, respectively, when cultured in the 60MME medium (Fig. 4b), probably attributed to the rich aspartate dosage. Both mutants revealed that deletions of doeA and ectD had little effect on their growth (Supplementary Fig. 4), this is helpful for scale-up studies on co-production of PHB and ectoine.

Chromosomally transcriptional tuning of ectABC. Chromosome integration of target expression module(s) allows robust performance of cell growth under antibiotic-free cultivation. Therefore, a GFP-mediated transcriptional mapping approach developed for fine-tuning target genes in the previous study was employed to screen an appropriate constitutive promoter for chromosomal expression of the ectABC cluster (Fig. 5a). Specifically, fluorescent protein sfGFP serving as a global reporter for expression levels was applied to quantify and bridge the correlations cross various promoters and expression systems for efficiently screening the suitable promoter used to transcribe target expression modules, such as ectABC, respectively (Supplementary Fig. 5). In this study, the T7-like induced system was characterized again by expressing sfGFP (Fig. 6b). Simultaneously, a porin promoter library was constructed and characterized by expressing sfGFP as described in our previous study.

Since the optimized expression strength of ectABC depends on the T7-like induced system, such as the optimized 20 mg L⁻¹ IPTG induction for the T7-like system, yet IPTG is an expensive chemical not suitable for large scale applications. Thus,
a constitutive porin promoter mutant P140, which exhibits a proximal expression level compared to the T7-like promoter induced by 20 mg L\(^{-1}\) IPTG in terms of the fluorescence intensity values (Fig. 6b and Supplementary Table 3), was selected to express ectABC module on the chromosome of *H. bluephagenesis* TD-AD (G4 loci) (Fig. 5a), forming *H. bluephagenesis* TD-ADE strain. However, the ectABC controlled by a stronger promoter with a fluorescence intensity value greater than 50,000 to achieve a high expression had failed to construct in *E. coli* (also for conjugation purpose). Since the expression levels of target modules on plasmid pSEVA321 are reported to be 9-fold higher than that of the chromosomal expression, which can easily lead to poor- or non-growth due to the overload of target fluxes in donor cells (*E. coli* S17-1)\(^{38}\). Ectoine production by ectABC fine-tuned *H. bluephagenesis* TD-AD was carried out in shake flasks containing both 60MME and 60MMU media, forming 4.7 g L\(^{-1}\) and 4.3 g L\(^{-1}\) ectoine, respectively (Fig. 5b and c). In contrast, *H. bluephagenesis* TD1.0 harboring ectABC expression module under T7-like promoter encoded on a plasmid-based expression system was used as positive control termed *H. bluephagenesis* TD-AD-E, produced approximately 4 g L\(^{-1}\) ectoine, significantly lower than 4.6 g L\(^{-1}\) ectoine produced by the *H. bluephagenesis* TD-AD containing the chromosome-based ectABC system under the control of porin promoter mutant P140, yet much higher than the control *H. bluephagenesis* TD-AD strain forming only less than 2 g L\(^{-1}\) ectoine (Fig. 5b, c).

**Fine-tuning of lysC and asd in genome of *H. bluephagenesis*.** *H. bluephagenesis* TD-ADE containing the chromosome-based and fine-tuned ectABC system under the control of porin promoter mutant P140 has enhanced the production of ectoine (Fig. 5). It is expected that further flux-tuning of *asd* and *lysC* genes encoding L-aspartate-semialdehyde-dehydrogenase and aspartokinase, respectively, could channel more metabolic fluxes toward ectoine synthesis (Fig. 1)\(^{16,49}\). Therefore, gene lysC of *Corynebacterium glutamicum* and *asd* of *H. bluephagenesis* were overexpressed in *H. bluephagenesis* TD-ADE, followed by transcription tuning to obtain better performance on ectoine accumulation.

Two inducible promoters are required including T7-like promoter to modulate the expression of *lysC* and *asd* genes, simultaneously and respectively. In this case, a quorum sensing-based regulator LuxR encoded by a luxR triggering the transcription of the corresponding promoter P\(_{lux}\) in the presence of acyl homoserine lactone (AHL) was constructed on a plasmid-based expression system controlled by the constitutive promoter P\(_{lac}\)\(^{100}\), together with sfGFP controlled by P\(_{lux}\) for the dose-response functional characterization in *H. bluephagenesis* TD-ADE using flow cytometer analysis (Fig. 6a and Supplementary Fig. 6). Results exhibited 86-folds dynamic range of fluorescence intensity (FI) of sfGFP in the presence of various AHL concentrations with low basal leakiness, FI = 157 (Supplementary Fig. 6). Subsequently, the expression module of P\(_{lac}\)luxR was integrated in G43 loci in *H. bluephagenesis* TD-ADE, forming *H. bluephagenesis* TD-ADE, forming *H. bluephagenesis* TD-AD-E.
**Fig. 4 Effect of deleting ectoine degradation genes doeA and/or ectD on ectoine synthesis.**

**a** Primer design and colony PCR results of doeA and ectD deletion for blocking the probable degradation of ectoine. **b, c** Performances of ectoine accumulation by various engineered *H. bluephagenesis* TD1.0 strains, respectively, strain TD1.0: a start host, strain TD-A: single-deletion of doeA, and strain TD-AD: double-deletion of doeA and ectD genes. Strains harboring the ectABC expression module on plasmid-based systems, namely, strain TD1.0-E, TD-A-E, and TD-AD-E, respectively, induced with 20 mg L\(^{-1}\) IPTG. **b** Cells cultured in 60MME medium, while (c) was grown in 60MMG medium supplemented with extra 3 g L\(^{-1}\) urea (60MMU) for de novo biosynthesis of ectoine from glucose and urea only. Data are presented as mean values, error bars represent standard deviations (SD); \(n = 3\) biologically independent samples. Source data are provided as a Source Data file.

**Fig. 5 Chromosomal expression of ectABC in *H. bluephagenesis* TD1.0 for ectoine production.**

**a** Transcription-tuning design based on stimulus responsive flux-tuning approach mediated by sfGFP fluorescence intensity. Reprinted from Metabolic Engineering, 57, Jianwen Ye, Dingkai Hu, Jin Yin, Wuzhe Huang, Ruijuan Xiang, Lizhan Zhang, Xuan Wang, Jianing Han, Guo-Qiang Chen, Stimulus response-based fine-tuning of polyhydroxyalkanoate pathway in *Halomonas*, 85–95, Copyright (2020), with permission from Elsevier. **b, c** Shake flask results of ectoine production by recombinant *H. bluephagenesis* TD1.0 (TD-AD) and its derivates with ectABC expression on plasmid-based system in the presence of 20 mg L\(^{-1}\) IPTG (TD-AD-E) and chromosome-based system under the control of porin promoter mutant P\(_{140}\) (TD-ADE), respectively. **b** Cells cultured in a 60MME medium, while (c) were grown in the 60MMU medium for de novo biosynthesis of ectoine. Data are presented as mean values; error bars represent standard deviations (SD); \(n = 3\) biologically independent samples. Source data underlying (b, c) are provided as a Source Data file.
bluephagenesis TD-LuxR strain (Fig. 6a). 275- and 1843-fold dynamic ranges were obtained from H. bluephagenesis TD-LuxR under the LuxR-AHL and T7-like induced systems, respectively, the basal leakiness of chromosome-based LuxR-AHL induced system decreased to 10 FI value (Fig. 6b). Thus, two orthogonal induced systems were successfully constructed in H. bluephagenesis enabling double-module modulation (Fig. 6c).

Ideally, expression of lysC and asd would be induced by IPTG and AHL, respectively, for enhanced ectoine production by consuming more urea. Before that, it is necessary to optimize urea concentration in the culture medium again. Addition of 5 g L\(^{-1}\) of urea to the 60MMG medium (namely 60MMU5) generated 17% improvement on ectoine accumulation although cell growth decreased significantly (Fig. 6d). Here, two expression modules, namely, asd and lysC controlled by Plux and PT7-like, respectively, were constructed on a plasmid-based system for transcriptional tuning (Fig. 6e). The induction concentrations of IPTG and AHL did have a significant effect on ectoine production. For example, an induction using 20 mg L\(^{-1}\) IPTG and 10 mg L\(^{-1}\) AHL generated the highest 6.6 g L\(^{-1}\) ectoine, 29% higher compared to that produced by H. bluephagenesis TD-ADE (Fig. 6d, f, Supplementary Fig. 7). Using the GFP-mediated transcription-tuning approach,
porin promoter mutants P226 and P140 were selected to control the expression of lysiC and P98 and P183 for asd on the chromosome of H. bluephagenesis TD-ADE.

However, the expression module encoding lysiC controlled by P140 failed to construct in the donor E. coli as also mentioned previously. Yet, two strains, H. bluephagenesis TD-ADE-58 and TD-ADEL-183, were successfully grown for shake flask studies using the 60MMU5 medium (Fig. 6g). The two strains produced almost 6.3 g L⁻¹ ectoine after 48 h cultivation in the 60MMU5 medium, over 30% higher compared to that cultured in the 60MMU medium. Due to the failure of P140-lysC construction, a weaker transcription strength of lysiC controlled by P226, probably weakens the flux for L-β-aspartyl phosphate synthesis, which coupled with asd controlled by P183, it cannot bring significant increase on ectoine when grown in both 60MMU and 60MMU5 media, respectively. Thus, H. bluephagenesis TD-ADE-58 became a better option for fed-batch studies in 7 L lab-scale bioreactor. Similarly, considering the fed-batch study for co-production of PHB and ectoine, H. bluephagenesis TD-ADE with genomic expression of lysiC and asd genes were performed in a 96-well plate to investigate the effect on growth scale-up (Supplementary Fig. 4).

Fed-batch study for co-production of PHB and ectoine. H. bluephagenesis TD-ADE-58 was grown for 28 h for ectoine production under open unsterile conditions in a 7 L bioreactor. 28 g L⁻¹ ectoine were produced from this fed-batch study, which is over 4-folds higher than that of the shake flask results (Fig. 7a). The yield of ectoine on glucose and volumetric productivity reached approximately 0.21 g g⁻¹ and 1.0 g L⁻¹ h⁻¹, respectively. Ectoine accumulation tendency was highly consistent with cell growth in terms of DCM, indicating the growth-coupled property of ectoine biosynthesis. Meanwhile, PHB accumulation was maintained at a low level during the first 16 h of cultivation before its increase afterward (Fig. 7a). In contrast, a fed-batch fermentation was executed for 28 h using H. bluephagenesis TD-ADE to verify the effect of fine-tuning asd and lysiC gene on ectoine production, leading to 17 g L⁻¹ ectoine, which was over 30% lower than results of H. bluephagenesis TD-ADE-58 (Supplementary Fig. 8).

To achieve co-production of ectoine and PHB by H. bluephagenesis TD-ADE-58, a tailor-made feeding solution was designed to enhance PHB accumulation during the stationary phase using reduced nitrogen dosage in the second phase feeding solution (Feed-II). Similarly, a fed-batch fermentation was carried out in the 7 L bioreactor to co-produce ectoine and PHB in different growth phases. After a 44 h cultivation, the DCM reached 32 g L⁻¹ containing 75% PHB, together with 8 g L⁻¹ ectoine (Fig. 7b). It was interesting to observe that ectoine biosynthesis maintained active in the stationary phase during the fed-batch cultivation (Fig. 7b). It is expected that more bioprocess optimizations should lead to even higher ectoine and PHB production under balancing nitrogen supply in different growth phases.

Discussion

Next generation industrial biotechnology (NGIB) based on halophiles provides low-cost and convenient processing conditions for production of chemicals. The most successful case has been exemplified by 5000 L scale fermentor for PHA production. It has been interesting to look for other examples to test possibilities of NGIB for production of non-polymer products, or even high-value added example. Ectoine, a high-value added product derived from L-aspartate synthesized by some microorganisms, has attracted increasing attentions due to its outstanding functions of stabilizing proteins and nucleic acids. To date, many ectoine commercial applications, such as cosmetic ingredient, have been developed with high-value. However, ectoine production by wild type and engineered microorganisms yields very low productivity. Some metabolic engineering approaches have been adopted for enhanced productions of amino acids-related compounds, including ectoine, l-threonine and l-lysine, including new tools for strain engineering, such as dynamic and static optimization for flux enforcement, stimulus response-based transcriptional controls and promoter and RBS optimizations et al. H. bluephagenesis, a high-salt tolerant halophile able to grow to a high cell density with scalability, is a promising host for ectoine synthesis because of its being a natural ectoine producer harboring the ectABC cluster in its genome. Recently, many efforts have been made to establish various genetic manipulation tools and flux-tuning approaches for improving and diversifying this NGIB chassis. This study has demonstrated successful and high yield on ectoine production by chromosomally engineered H. bluephagenesis utilizing static optimization strategies, reaching close to 30 g L⁻¹ ectoine from a fed-batch fermentation process (Fig. 7a).

Ectoine biosynthesis pathway of H. bluephagenesis was clearly identified by heterologous expression in recombinant E. coli, in which strong enzymatic activities of ectABC cluster were found compared with previous studies. Cell growth studies uncovered the enhanced resistance of cells under both low- and high-salt concentrations in the presence of ectoine,
respectively. Furthermore, competitive ectoine degradation pathways were removed by eliminating the related genes doaA and ectD, resulting in 89% improvement on ectoine production from shake flask cultivations. Two pathway branches reported by previous studies involved in L-threonine and L-lysine synthesis were retained in consideration for the benefits of ectoine co-production with PHB. A growth-dependent intracellular product, as the disruption of L-threonine and L-lysine will weaken cell growth and thus PHB accumulation. We thus attempted to utilize transcriptional balancing strategies to boost fluxes towards ectoine synthesis. In contrast to translational regulation, transcriptional regulation could be more efficient to be carried out with minor constructs during strain engineering by one-step fine-tuning of target expression modules based on stimulus response-based flux-tuning design.

Five genes involved in ectoine synthesis pathway were grouped into three individual expression units, including ectABC, lacC, and asd, for transcriptional fine-tuning (Figs. 1, 4). It has to be noted that quorum-sensing-based inducible system, LuxR-AHL, was firstly constructed and employed to achieve combinatorial transcriptional tuning together with T7-like system in H. bluephagenesis (Figs. 5, 6). Specifically, expression of two simple constructs, p321-PJ23110-lacI-P-T7-like-ectABC and p321-P-T7-like-lacC-P-lux-asd, could simulate countless transcriptional combinations of target modules replacing thousands of variants construction. Finally, 9.8-folds improvement of production titer of ectoine was achieved in a shake flask study by recombinant H. bluephagenesis harboring fine-tuned synthesis pathway of ectoine in genome compared with 0.64 g L\(^{-1}\) titer yielded by the start host (Fig. 6). Subsequently, an ectoine titer of approximately 30 g L\(^{-1}\) was achieved under open unsterile fed-batch fermentation conducted in the 7-L bioreactor.

Co-production of PHB and ectoine allows harvests of intracellular bioplastic PHB and extracellular small molecular chemical ectoine, it is thus economically interesting. However, it is not sure if the two products competing for metabolic fluxes could reduce their yields. A conceptional proven study was conducted in a lab-scale fermentation. Both ectoine and PHB are growth-associated products derived from aspartate and acetyl-CoA when nitrogen source supply is abundant, approximately 28 g L\(^{-1}\) ectoine and 25 g L\(^{-1}\) DCM together with 6% PHB were obtained, respectively, during a 28 h of growth (Fig. 7a). In contrast, under a reduced nitrogen source, PHB accumulation was increased to 75% of the DCM in the cells producing also 8 g L\(^{-1}\) ectoine (Fig. 7b). In both cases, the recombinant H. bluephagenesis was grown to over 30 g L\(^{-1}\) DCM that are not the highest reported by this organism. Further efforts to increase the recombinants’ growth density should lead to much improved ectoine and PHB productions.

Ectoine as a representative product of L-aspartate compound family with a high value, should be able to produce at a competitive cost after fine-tuning of every single gene of ectABC cluster employing SR-FT approach by developing more suitable inducible systems. Although static optimization is a widely used strategy for enhanced production of metabolic targets, recent progresses focused on bifunctional dynamic control provide a strong hint for improving the production of ectoine. Since co-production of PHB and ectoine seems to be competing for flux, the efficacy to activate target pathways dynamically for PHB and ectoine must be considered during the fermentation processes. In addition, the bioprocessing optimization, such as feeding solution optimization and cascaded bioprocessing, is a promising strategy to obtain simultaneously enhanced production yields for ectoine and PHB. Additionally, attempts of enhancing export system and developing amino acids responsive regulators could also be applied to achieve further improvement on ectoine and PHB production under cost-effective bio-manufactural process based on the NGIB chassis.

In conclusion, the halophilic NGIB chassis can be effectively used for effective production of ectoine. Engineering the ectoine pathways improved ectoine production effectively. Co-production of intracellular PHA and non-polymer chemicals is possible using the halophilic chassis.

**Methods**

**Strains culture and chemicals.** LB medium is composed of (g L\(^{-1}\)): 10 trypton, 5 yeast extract and 10 NaCl. 60 LB medium is derived from LB medium supplemented with 1 g L\(^{-1}\) NaCl. 60MMG medium consists of (g L\(^{-1}\)): glucose 30, NaCl 60, yeast extract 1, (NH\(_4\))\(_2\)SO\(_4\) 0.25, MgSO\(_4\) 0.2, Na\(_2\)HPO\(_4\) 0.125, KH\(_2\)PO\(_4\) 1.5, trace element solution I 10 mL L\(^{-1}\) and trace element solution II 1 mL L\(^{-1}\). The composition of trace element solution I consists of: Fe(III)-NH\(_4\)-citrate 5, CaCl\(_2\) 2, HCl 1 M. The trace element solution II (mg L\(^{-1}\)) consists of: ZnSO\(_4\)·7H\(_2\)O 100, MnCl\(_2·4H\(_2\)O\) 30, K\(_2\)HPO\(_4\) 300, CoCl\(_2·6H\(_2\)O\) 200, CuSO\(_4·5H\(_2\)O\) 10, NiCl\(_2·6H\(_2\)O\) 20, and NaMoO\(_4·2H\(_2\)O\) 30. 60MME is derived from 60MMG medium supplemented with 100 mM sodium aspartate with additional 100 mM KCl, and 1.7 g L\(^{-1}\) citrus acid. Alternatively, 100 mM sodium aspartate in 60MME medium was replaced by 3 and 5 g L\(^{-1}\), respectively, to form 60MMU and 60MMUS media for de novo synthesis of ectoine for shake flask studies. E. coli strains TD-1 harboring plasmid p321-PJ23110-LacI was transformed into recombinant H. bluephagenesis TD were cultivated in 60MMG, 60MME, 60MMU and 60MMUS media, respectively.

All the derivates from H. bluephagenesis TD1.0, wild type H. bluephagenesis TD integrated with expression module of MmpR PRR polymerase induced by IPTG, were listed in Supplementary Table 1. Firstly, doaA and ectD gene encoding bypass (ectoine degradation) enzymes were deleted, forming H. bluephagenesis TD-AD strain. Then, expression modules including ectABC, lacC and asd cluster were integrated into the chromosome of H. bluephagenesis one-by-one at G4, G7 and G43 locus, respectively, forming H. bluephagenesis strains TD-AD-E, TD-AD-E-L, TD-AD-58, and TD-AD-183.

**Plasmids construction.** Plasmids were constructed to carry functional gene expressions encoded in vector pSEVA321. All the constructs were assembled by Gibson Assembly kit, and conjugated into H. bluephagenesis derivatives. DNA extraction was carried out by using Tiangen Plasmid Extraction Kit (Shanghai, China) and Omega Gel Purification Kit (USA). DNA oligonucleotides were synthesized by Zixi Biotechnology (Beijing, China). Genetic design and sequence reading were performed by Snap Gene (v2.3.2).

All the plasmids were constructed by Gibson Assembly kit. For gel purification, the Omega gel purification kit was used, and the Tiangen plasmid extraction kit was used to obtain the plasmids. All the plasmids used in this study and genes required fine-tuning to obtain enhanced ectoine production were listed in Supplementary Table 1. The sequences of sgRNA used for genome editing in the study were listed in Supplementary Table 2. CRISPR-based genomic editing approach was employed to construct various recombinant strains by gene knockdown and gene integration. Firstly, plasmid pQ08 containing Cas9 expression module purchased from Sigma-Aldrich (St. Louis, MO, USA) was used as standard for HPLC analysis for fermentative ectoine quantitation.

**Conjugation.** Firstly, plasmids were constructed by E. coli DH5α, subsequently transformed into E. coli S17-1, the donor strain for conjugation. Then, donor cells harboring target constructs were cultivated for 12–14 h in 150-mL shake flasks containing 20 mL LB medium, and recipient cells, recombinant H. bluephagenesis, were cultivated in 60 LB medium overnight. Both of the donor and recipient cells were centrifuged and harvested under 4°C, washed with a fresh medium and recovering to the same volume that sampled. Subsequently, cells were mixed (1:1 volume) and dropped on antibiotic-free 20-LB agar plates, followed by 6–8 h incubation at 37°C for conjugation. Finally, conjugated cells from the lawn were suspended with 60 LB medium and spread on 60 LB agar plates containing relevant antibiotics for 36 h incubation to obtain positive colonies.

**Characterization of LuxR-AHL and T7-like inducible systems.** LuxR-AHL inductive system was first constructed on plasmid-based expression system in H. bluephagenesis TD1.0 harboring plasmid p321-PJ23110-lacI-P-T7-like- to characterize the performance in the presence of various AHL (OC6, from Sigma)
concentrations, respectively. Then, luxX expression module was integrated into the chromosome of H. bluephagenesis TD-ADE at G43 loci, forming TD-LuxX strain, following the characterization of LuxR-AHL and T7-like induced systems in H. bluephagenesis TD-UX strain harboring plasmids p321-PutX usurped p321-Pr7-like-sgfp after conjugation. Cells carrying target expression vessels were plated on 60 LB agar plate added with relevant antibiotics for 16–20 h of incubation at 37 °C. Subsequently, single colony was inoculated into 1 mL 60 LB medium for 12 h of cultivation. 96-deep-well plates were covered with sealing films (BM-400-S; Thermo-Shaker, Aosheng, 37 °C, 1000 rpm). Then, 5 µL cell culture was inoculated into a new plate supplemented with appropriate antibodies in presence of a spectrum of inducer. After 12 h incubation, cell cultures were diluted 250-folds using phosphate-buffered saline solution (PBS) for flow cytometry analysis. Positive fluorescent cells were recorded by flow cytometer (LSRFortessa, BD bioscience, USA) at the rate of 0.5 µL s⁻¹ for 20 s with at least 30,000 cell captured in total of each sample, and the start host, H. bluephagenesis TD1.0, was used as the negative control groups to modify the fluorescence levels of experimental groups. Intracellular sfgfp protein was excited under 488 nm, and cells were captured on the signal channels of FITC, FSC (scatter) and SSC (side scatter). FlowJo (version 7.6) software was used to process the raw data for obtaining mean value of fluorescence intensity and percentage of positive cells.

Cell growth study. To study the effects of ectoine and NaCl on cell growth, single colony of recombinant H. bluephagenesis TD1.0 was first inoculated for an incubation of 12 h incubation in 1 mL 60 LB medium in a 96-deep-well plate (Corning/USA, 1 mL 60 LB medium) at 37 °C. Subsequently, seed cultures were diluted 200-folds with fresh MM medium supplemented with 30 g L⁻¹ glucose and nitrogen source (urea or sodium aspartate) whenever necessary. 15 µg mL⁻¹ chloramphenicol was added to the medium grown with H. bluephagenesis harboring the plasmid-carried expression systems. Cell cultures were harvested for dry mass (DCM), ectoine and PHB content assays after 48 h cultivation. Additionally, functional verification of gene cluster etABC from H. bluephagenesis was performed using the same procedure of recombinant H. bluephagenesis grown in LB medium and recombinant E. coli DH5α harboring plasmid p321-Pr7-like-sgfp-putX-etcABC.

Shake flask and fed-batch studies. For shake flask studies, recombinant H. bluephagenesis harboring target expression modules on plasmid- or chromosome-based systems were pre-cultured overnight to obtain seed cultures (OD₆₀₀ reached 2.5 ± 0.2) from a single colony, respectively. Subsequently, 2.5 mL seed culture were inoculated into 50 mL 60 MM medium supplemented with 30 g L⁻¹ glucose and nitrogen source (urea or sodium aspartate) whenever necessary. 25 µg mL⁻¹ chloramphenicol was added to the medium grown with H. bluephagenesis harboring the plasmid-carried expression systems. Cell cultures were harvested for dry mass (DCM), ectoine and PHB content assays after 48 h cultivation. Additionally, functional verification of gene cluster etABC from H. bluephagenesis was performed using the same procedure of recombinant H. bluephagenesis grown in LB medium and recombinant E. coli DH5α harboring plasmid p321-Pr7-like-sgfp-putX-etcABC for 12 h by picking a single colony as an inoculum. Then, seed cultures were diluted 200-folds with a fresh 60 LB medium in a new 96-well plate (flat bottom, 250 µL medium per well) for online monitoring of optical density at 600 nm (OD₆₀₀) for 48 h. To characterize cell growth of recombinant H. bluephagenesis in this study, chromosomally engineered strains were pre-cultured in a 60-LB medium in the 96-deep-well plate for 12 h by picking a single colony as an inoculum. Then, seed cultures were diluted 200-folds with a fresh 60 LB medium in a new 96-well plate (flat bottom, 250 µL medium per well) for online monitoring of optical density at 600 nm (OD₆₀₀) for 24 h. The cell growth characterization was carried out using a micro-plate reader (Varioskan Flash, Thermo Scientific).

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Author contributions
G.Q.C. and J.Y. proposed the idea; G.Q.C. and J.Y. designed and supervised the experiments; H.M. performed the HPLC-MS/MS experiments and medium optimization; Y.Z. performed the genome editing experiments and the tests; H.M., Y.Z., W.H., and L.Z., contributed to the experimental research; H.M., Y.Z., W.H., and L.Z., performed the fermentation experiments; H.M., Y.Z., and F.W. performed all other experiments; H.M., Y.Z., J.Y., and G.Q.C. analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

Competing interests
The authors declare no competing interests.
**Additional information**

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