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Inducible Population Quality Control of Engineered *Bacillus subtilis* for Improved *N*-Acetylneuraminic Acid Biosynthesis

Yanting Cao, Rongzhen Tian, Xueqin Lv, Jianghua Li, Long Liu, Guocheng Du,* Jian Chen, and Yanfeng Liu*

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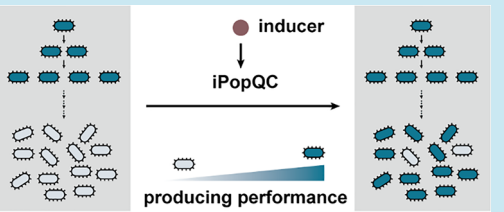
ABSTRACT: Biosynthesis by microorganisms using renewable feedstocks is an important approach for realizing sustainable chemical manufacturing. However, cell-to-cell variation in biosynthesis capability during fermentation restricts the robustness and efficiency of bioproduction, hampering the industrialization of biosynthesis. Herein, we developed an inducible population quality control system (iPopQC) for dynamically modulating the producing and nonproducing subpopulations of engineered *Bacillus subtilis*, which was constructed via inducible promoter- and metabolite-responsive biosensor-based genetic circuit for regulating essential genes. Moreover, iPopQC achieved a 1.97-fold increase in *N*-acetylneuraminic acid (NeuAc) titer by enriching producing cell subpopulation during cultivation, representing 52% higher than that of previous PopQC. Strains with double-output iPopQC cocoupling the expression of double essential genes with NeuAc production improved production robustness further, retaining NeuAc production throughout 96 h of fermentation, upon which the strains cocoupling one essential gene expression with NeuAc production abolished the production ability.



KEYWORDS: *biosensor, genetic circuit, cell growth and product synthesis coupling, inducible population quality control, Bacillus subtilis, N-acetylneuraminic acid*

Synthetic biology-driven construction of microbial cell factories for producing high value-added products has become an important approach for the green and sustainable biomanufacturing of chemicals.[1−4] However, due to environmental and cellular variations, such as differences in local conditions within the same bioreactor, stochastic gene expression, copy number variation, and genetic heterogeneity and other factors, a cell population generated from a single colony can display cell-to-cell variation in growth and metabolism, for instance, the generation of producing a cell subpopulation that involves a high-performing cell subpopulation and a low-performing cell subpopulation, as well as a nonproducing cell subpopulation.[5−10] The producing cell subpopulation usually shows reduced growth fitness, especially for a high-performing cell subpopulation, due to the production burden resulting from competition between the endogenous cell metabolisms and heterologous pathways, as well as the toxicity of intermediate products, end products, or expressed heterologous proteins.[11−13] During long-term or large-scale fermentation, the producing cell subpopulation can be gradually replaced by a rapidly growing nonproducing cell subpopulation that consumes nutrients for cell growth without synthesizing target products, thereby reducing the robustness and efficiency of production and restricting the industrial applicability of biosynthesis.[4,14−16]

To address this problem, population quality control systems have been developed to enrich the producing cell subpopulation of engineered cells during fermentation.[17−19] They use positively product-responsive biosensors to regulate the expression of cell growth determinant genes, such as antibiotic-resistant genes (culturing with antibiotic addition), key genes in the amino acid synthesis pathway, or essential genes, thereby producing cells or high-performing cells with activated expression of cell growth. Determinant genes can maintain normal growth, whereas nonproducing cells show a decreased growth with the inhibited expression of cell growth determinant genes. By this way, population quality control confers a growth advantage on producing or high-performing cell subpopulations, thereby enhancing the biosynthesis efficiency or production robustness of fatty acids, tyrosine, mevalonic acid, and naringenin by enriching the producing or high-performing cell subpopulation during cultivation.[17−19] However, potential restrictions on the applicability and robustness of previous population quality control systems may limit the application and efficiency of cell subpopulation control. For applicability, in the nonproducing phase, such as preparing competent cells or seed cultures, the target product

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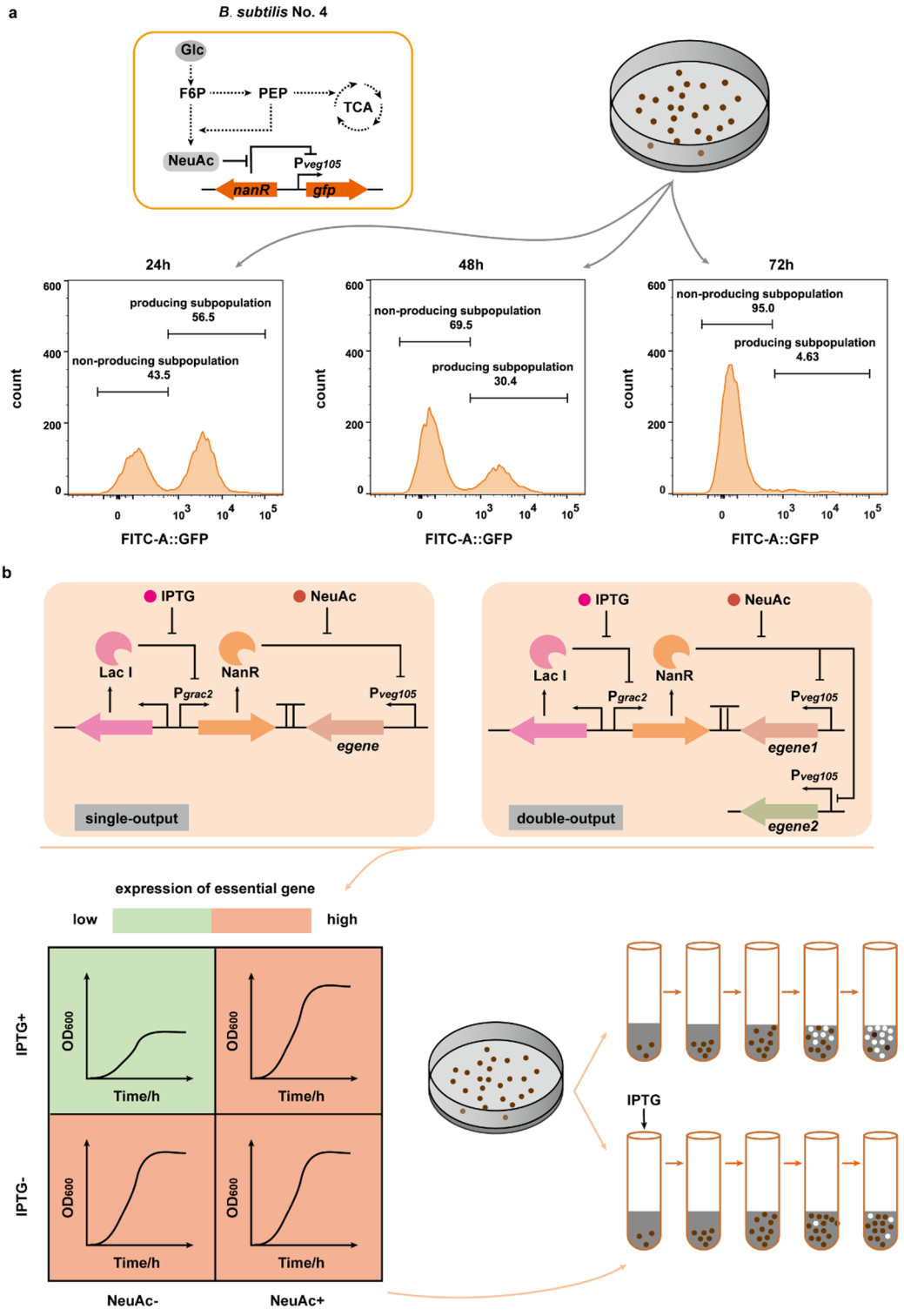

Figure 1. Cell-to-cell variation in NeuAc biosynthesis and design of iPopQC systems for enriching the NeuAc-producing cell subpopulation. (a) Population generated from a single NeuAc-producing colony involved both producing and nonproducing subpopulations. Single colonies of NeuAc-producing strain No. 4, which carried the NeuAc-responsive biosensor, were cultivated in 24-well deep plates. At different time points, cultures were collected and analyzed by flow cytometry. Nonproducing strain No. 3, which also carried the NeuAc-responsive biosensor, was used as a control to gate the nonproducing cell subpopulation. (b) Design of iPopQC systems involving the single output (cocoupling one essential gene expression with NeuAc production) and double output (cocoupling two essential gene expressions with NeuAc production). Essential gene (egene) is regulated by an inducible NeuAc-responsive biosensor. In the absence of an inducer, transcriptional factor NanR expression is inhibited whereby the essential gene expression level is relatively high, rendering normal growth. In the presence of inducers, cells with intracellular NeuAc have a relatively high level of essential gene expression, showing almost normal growth. However, cells without intracellular NeuAc have a relatively low level of essential gene expression, displaying inhibited growth. By this way, the producing cell subpopulation (dark color) is conferred a growth advantage and can be enriched during cultivation. In the meantime, IPTG acts as an inducer to functionalize iPopQC, achieving dynamic control of the producing (dark color) and nonproducing subpopulations (light color) of engineered *B. subtilis*.

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must be added to the medium to maintain normal growth of cell while cocoupling the expression of the essential gene to the product synthesis, which is costly and only suitable for the product that can be transported from the medium into the cell. However, blocking the product uptake often facilitates extracellular product accumulation in metabolic engineering,[20,21] which further restricts the applicability of previous population quality control systems. Despite selection from previous antibiotic-based systems that were induced by adding antibiotics to initiate a population quality control system, antibiotic addition is not favorable for food grade product biosynthesis.[17] Moreover, use of antibiotics is also environmentally problematic.[17] For robustness, while using previous population quality control systems, the product-responsive promoter is used to control the expression of growth determinant genes in one expression cassette; therefore, during long-term and large-scale fermentations demanding more cell divisions, the population quality control systems may be susceptible to losing functionality when mutations occur in gene sequences of the product-responsive promoter.[18]

In this study, we developed an inducible population quality control system (iPopQC) for dynamically modulating the producing and nonproducing subpopulations of engineered *Bacillus subtilis* during fermentation, which does not require product addition during the nonproducing phase and is applicable for both kinds of metabolites that can or cannot be transported into the cells. iPopQC was constructed via an inducible promoter- and metabolite-responsive biosensor-based genetic circuit that controls the expression of essential genes. Moreover, we test the effects of iPopQC on *N*-acetylneuraminic acid (NeuAc) production and cell subpopulation enrichment during fermentation. Finally, by cocoupling the expression of double essential genes with NeuAc production, we constructed double-output iPopQC to be more robust than single-output iPopQC. We cocoupled one essential gene expression with NeuAc production, and then tested its performance in improving production robustness in long-term fermentation and large-scale fermentation mimicked by serial passaging.

RESULTS AND DISCUSSION

Cell-to-Cell Variation in NeuAc Synthesis Performance. *B. subtilis* is a Gram-positive model bacterium and an important industrial microorganism that has long been used for the microbial production of important enzymes and biochemicals, especially nutraceuticals, due to its generally regarded as safe status.[22−25] Currently, a population quality control method is not available for *B. subtilis*. NeuAc is an important monomer of sialylated human milk oligosaccharide, which can be synthesized by engineered *B. subtilis* from glucose.[26−28] Therefore, we selected NeuAc production by *B. subtilis* as a case study for designing, building, and testing the iPopQC system.

We first aimed to reveal the cell-to-cell variation in NeuAc synthesis performance from NeuAc-producing strain No. 1 with 2.15 g/L of NeuAc titer, which was constructed by overexpressing the key genes related to NeuAc synthesis and blocking the competitive and branched pathways.[26,29] Metabolite-responsive biosensor-based flow cytometry has been broadly used to reveal cell-to-cell variation.[30] Therefore, we used a NeuAc-responsive biosensor, consisting of the transcriptional factor NanR and promoter veg105 (P_{veg105}) with the NanR-binding site, as well as the *gfp* reporter gene,[31] to reveal the distribution of cell subpopulations in NeuAc synthesis performance using flow cytometry. Under the control of this biosensor, *gfp* expression was inhibited without NeuAc and was activated in the presence of NeuAc (Figure 1a), but this biosensor showed the almost same output in the presence of 0.1 to 2 g/L of NeuAc.[31] Therefore, the NeuAc-responsive biosensor was used to analyze the distribution of the producing and nonproducing cell subpopulations in NeuAc synthesis performance. We next introduced the NeuAc-responsive biosensor[31] into NeuAc-producing strain No. 1, generating strain No. 4 (with NeuAc pathway and NeuAc-responsive biosensor). The nonproducing cell subpopulation was determined using strain No. 3 (without NeuAc pathway but with NeuAc-responsive biosensor) as a control, which shows the unimodal cell distribution with low GFP fluorescence intensity (Figure S1). The results show that the cell population generated from a single colony could be divided into nonproducing and producing cell subpopulations, and the proportion of producing cell subpopulation gradually decreased during cultivation (Figure 1a). To confirm that the different subpopulations were generated from cell-to-cell variations in NeuAc synthesis, not stability of the plasmid veg105-nanR6K, positive control was constructed by controlling *gfp* using the constitutive promoter P_{veg} in the same backbone as plasmid veg105-nonR6K (named pHvegG), and then the plasmid was transformed into NeuAc-producing strain No. 1. We found that the GFP fluorescence intensity of the positive control also showed unimodal cell distribution and almost uniform fluorescence intensity at 24, 48, and 72 h of cultivation (Figure S2). These results confirm the cell-to-cell variation in NeuAc synthesis performance.

Furthermore, on the basis of the cell growth curve and the proportion of nonproducing cell subpopulation at different growth stages, we constructed a mathematical model to explore the effect of a nonproducing cell subpopulation on NeuAc production. On the basis of the calculation results, when there is no nonproducing cell subpopulation during fermentation, the NeuAc titer can be 2.4 times of that with a gradually increasing nonproducing cell subpopulation during fermentation (Figure S3). These results suggest that cell-to-cell variation with existence of a nonproducing cell subpopulation is a challenge for efficient NeuAc biosynthesis.

Design Principle of iPopQC System. Although it is a core technology for realizing the green manufacturing of chemicals and a more sustainable future, biosynthesis is often subject to evolutionary pressures that select for nonproducing cells that display rapid cell growth and lower fitness cost, therefore impairing biosynthesis.[32−34] As demonstrated by our model above, reducing the proportion of nonproducing cell subpopulation in the culture can improve biosynthesis efficiency. Therefore, we designed iPopQC to dynamically modulate the producing and nonproducing cell subpopulations of engineered NeuAc-producing *B. subtilis* during fermentation (Figure 1b). iPopQC only functions in the presence of a chemical inducer by coupling cell growth with NeuAc synthesis using an NeuAc-responsive biosensor under the control of an inducible promoter to regulate the expression of an essential gene. In the absence of an inducer, the expression of the transcriptional factor NanR is inhibited, resulting in the expression of essential genes supporting normal cell growth, regardless of the presence of NeuAc. In the presence of an inducer, the expression of transcriptional factor NanR is activated, and it binds to P_{veg105} for inhibiting the expression of

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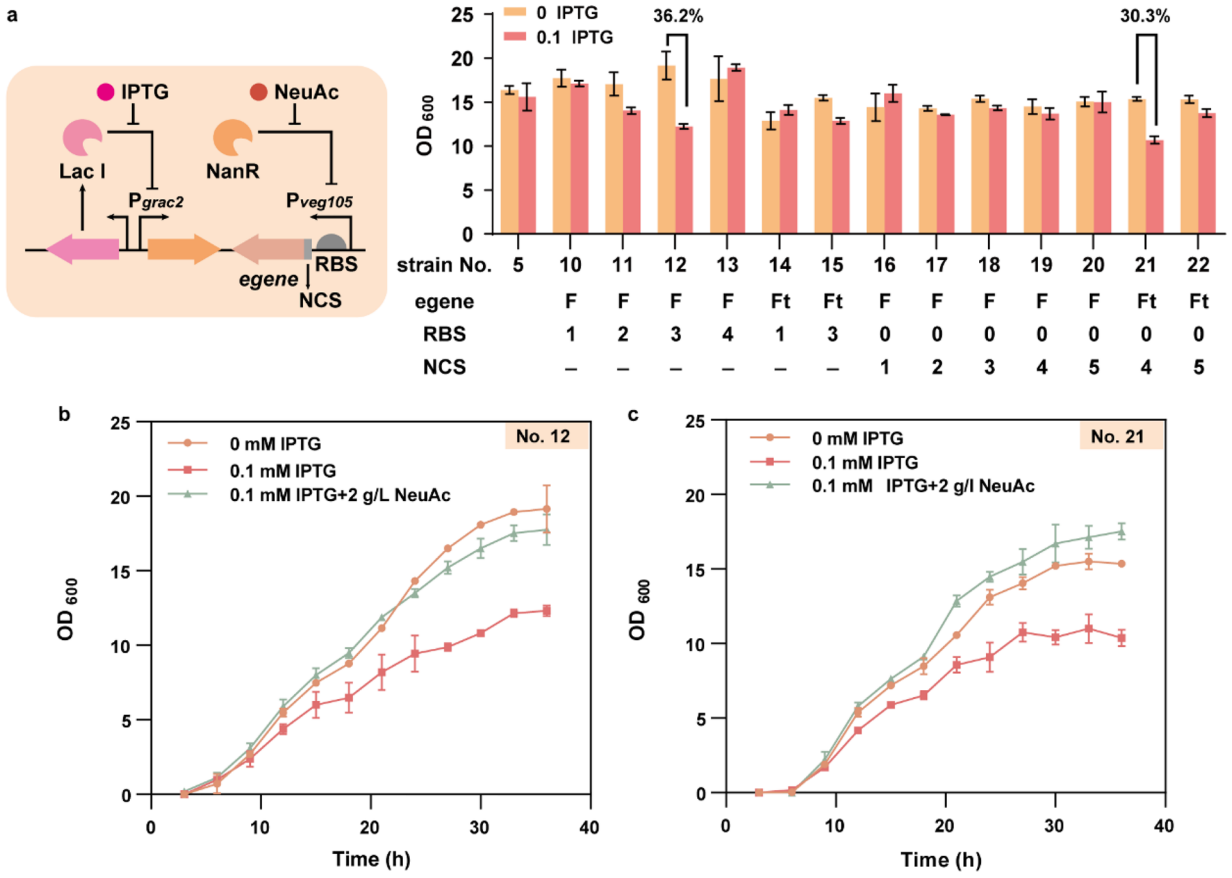

Figure 2. Characterization of iPopQC systems for coupling growth and synthesis. (a) Construction of strains Nos. 10−22 and their growth after 36 h of cultivation in the following different conditions: (1) adding 0 mM IPTG (0 IPTG) and (2) adding 0.1 mM IPTG (0.1 IPTG). Four different RBSs with different translation initiating rates and five different inhibitory NCSs with different intensities were used to regulate the expression of the essential genes *folB* encoding dihydroneopterin aldolase (F) and *ftsW* encoding cell division protein FtsW (Ft) in iPopQC in the level of translation to avoid the leakage expression of IPTG-inducible and NeuAc-responsive biosensor. All strains were derived from strain No. 5 by controlling essential genes *folB* or *ftsW* using IPTG-inducible NeuAc-responsive biosensor in the transcriptional level and the corresponding RBS or NCS (schemed in the bottom) in the translational level, followed by deleting the original copy of *folB* or *ftsW* in the genome. A single colony was inoculated in the fermentation medium in 24-well deep plates and cultivated at 37 °C and 220 rpm for measurements of OD_{600}. All data were the average of three independent studies with standard deviations. (b) Growth curve of strain Nos. 12 and 21 under different conditions, as follows: (1) addition of IPTG, (2) exclusion of IPTG, and (3) simultaneous addition of IPTG and NeuAc. Strain No. 12 was derived from strain No. 5 by controlling essential gene *folB* using the IPTG-inducible NeuAc-responsive biosensor in the transcriptional level and RBS3 in the translational level, followed by deleting the original copy of *folB* in the genome. Strain No. 21 was derived from strain No. 5 by controlling essential gene *ftsW* using the IPTG-inducible NeuAc-responsive biosensor in the transcriptional level and RBS0 as well as NCS4 in the translational level, followed by deleting the original copy of *ftsW* in the genome. Single colonies were inoculated in the fermentation medium in 24-well deep plates and cultivated at 37 °C and 220 rpm for measurement of OD_{600}. All data were the average of three independent studies with standard deviations.

essential gene, thereby causing decreased cell growth. The existence of intracellular NeuAc leads to the release of NanR that allows P_{veg105} to activate essential gene expression, recovering normal cell growth. Therefore, the inducer addition confers a growth advantage on the producing cell subpopulation. Compared to previous population quality control systems that are functional throughout cell growth, iPopQC, which only functions with an inducer addition, has the following two advantages: (1) there is no need to add product to the medium to maintain normal cell growth during the nonproducing phase, such as preparing competent cells or seed cultures, which is especially useful for products that cannot be taken up by engineered cells, such as NeuAc for *B. subtilis* (Figure S4). In contrast, the target product needs to be added to the medium to maintain normal cell growth while using the previous cell subpopulation control strategies. (2) With the use of a previous noninducible PopQC, cell growth may be affected by a low expression level of growth determinant genes, due to insufficient product synthesis during the early growth phase, which may further impair biosynthesis. However, iPopQC can mitigate this detrimental impact by dynamically initiating cell subpopulation control via changing the time of the addition of the inducer.

In addition, previous population quality control systems use the metabolite-sensing promoter to control the expression of growth determinant genes in one expression cassette, which may suffer dysfunction due to genetic mutations. Due to the existence of homologous recombination, nonhomologous recombination, insertion sequence element, plasmid instability, and intracellular and external environmental pressures, both engineered and wild cell types undergo continuous evolution at mutation rates from 10^{-2} to 10^{-10} per base pair per generation.[35] Genetic mutations in the sensor promoter may cause continuous expression of the cell growth determinant genes, invalidating population quality control systems. To avoid this, we designed a double-output iPopQC that uses the

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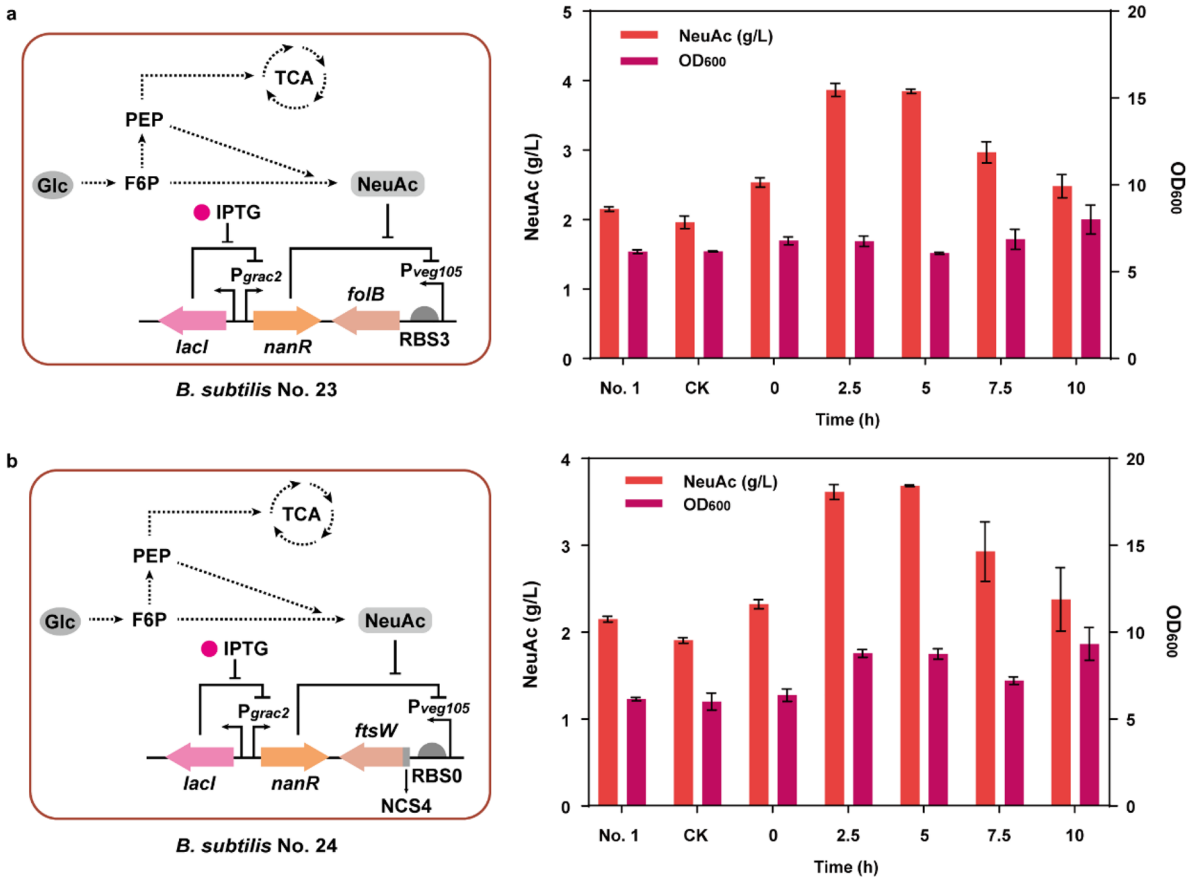

Figure 3. Validating iPopQC systems for improved NeuAc synthesis. (a) iPopQC mechanism and NeuAc production of strain No. 23. Strain No. 23 was constructed by introducing iPopQC systems in strain No. 12 (with essential gene *folB* regulated by sensor promoter P_{veg105} and RBS3) into NeuAc-producing strain No. 1. CK represents the control without IPTG addition. The times 0, 2.5, 5, 7.5, and 10 h represent that IPTG was added at 0, 2.5, 5, 7.5, and 10 h of cultivation. Single colonies were inoculated in the fermentation medium in 24-well deep plates and cultivated at 37 °C and 220 rpm for 60 h for measurements of OD_{600} and NeuAc titer. All data were the average of three independent studies with standard deviations. (b) iPopQC mechanism and NeuAc production of strain No. 24. Strain No. 24 was constructed by introducing iPopQC systems in strain No. 21 (with essential gene *ftsW* regulated by P_{veg105}, RBS0 and NCS4) into NeuAc-producing strain No. 1. CK represents the control without IPTG addition. The times 0, 2.5, 5, 7.5, and 10 h represent that IPTG was added at 0, 2.5, 5, 7.5, and 10 h of cultivation. Single colonies were inoculated in the fermentation medium in 24-well deep plates and cultivated at 37 °C and 220 rpm for 60 h for measurements of OD_{600} and NeuAc titer. All data were the average of three independent studies with standard deviations.

sensor promoter to regulate two essential genes in two expression cassettes (Figure 1b), in which iPopQC will be out of function only when mutations occur in both sensor promoters' upstream essential genes.[36,37] Therefore, a more stable double-output iPopQC cocoupling expression of two essential genes with NeuAc production is expected to be developed to further improve the robustness of production, which is a key challenge for long-term and large-scale industrial fermentation.

Characterization of iPopQC Systems for Coupling Growth to Synthesis. To construct iPopQC, isopropyl β-D-thiogalactoside (IPTG)-inducible promoter P_{grac2}, containing two LacI-binding sites, was used to control NanR expression (Figure S5a). Using GFP as a reporter, we next confirmed that the optimal IPTG concentration was 0.1 mM, at which the NeuAc-responsive biosensor had a maximal fold change (5.7-fold) between in the absence and presence of NeuAc (Figure S5b). To identify the essential genes for coupling cell growth to NeuAc synthesis, we separately controlled four essential genes (egene),[38] including *aspS* encoding aspartyl-tRNA synthetase, *dnaB* encoding replication initiation and membrane attachment protein, *folB* encoding dihydroneopterin aldolase, and *ftsW* encoding cell division protein FtsW, using the IPTG-inducible NeuAc-responsive biosensor. This was constructed by curing the NeuAc pathway plasmid pB6CG of No. 1 and the heterologously expressed NeuAc-transporter NanT that can import NeuAc from the medium to the cell. We next knocked out the original copies of the corresponding essential genes in the genome, resulting in the formation of strain Nos. 6−9. If cell growth is successfully coupled with NeuAc production, strains with 0.1 mM IPTG addition should display growth inhibition compared to those without IPTG in the medium without NeuAc, and growth recovery in the medium with NeuAc. Unexpectedly, all four strains showed similar growth with or without 0.1 mM IPTG in the absence of NeuAc (Figure S6b), which may have resulted from leaky expression of the NeuAc-responsive biosensor. Strains Nos. 8 and 9 maintained almost the same growth as the control strain No. 5 in the absence of 0.1 mM IPTG, in which iPopQC was not functioning, indicating that engineering of the expression of essential genes *folB* and *ftsW* showed limited fitness cost. Therefore, the essential genes *folB* and *ftsW* are suitable targets for coupling cell growth to synthesis.

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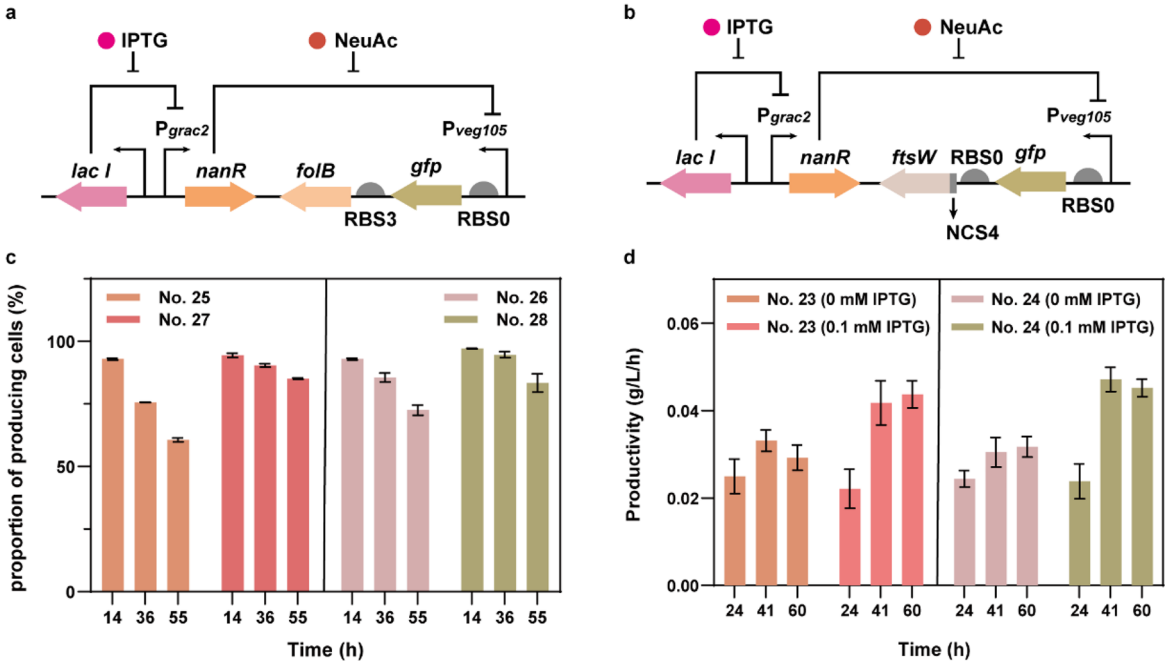

Figure 4. Enriched NeuAc-producing cell subpopulation by iPopQC. (a) iPopQC mechanism of strains Nos. 27 and 33. (b) iPopQC mechanism of strains Nos. 28 and 34. (c) Enriched producing cell subpopulations generated from single colonies of strains Nos. 27 and 28 by iPopQC. Strain No. 27 (No. 28) was constructed by introducing the iPopQC coexpressing *gfp* and *folB* regulated by RBS3 (iPopQC coexpressing *gfp* and *ftsW* regulated by RBS0 and NCS4) into producing strain No. 1. Strain No. 25 (No. 26) was taken as a control not coupling cell growth to NeuAc biosynthesis. The only difference between strains Nos. 25 and 27 (Nos. 26 and 28) was that strain No. 25 (No. 26) had an original copy of the *folB (ftsW)* gene in the genome. Cells were cultivated in the fermentation medium with 0.1 mM IPTG in 24-well deep plates at 37 °C and 220 rpm and collected at different time points for analyzing cell subpopulation distribution by flow cytometry. All data were the average of two independent studies with standard deviations. (d) Improved productivities by initiated iPopQC. Strains No. 23 (with essential gene *folB* regulated by P_{veg105} and RBS3) and No. 24 (with essential gene *ftsW* regulated by P_{veg105}, RBS0, and NCS4) were cultivated in the fermentation medium in 24-well deep plates at 37 °C and 220 rpm. IPTG was added at 0 h of cultivation when needed. Cells were collected at different time points for measurements of NeuAc titer. Productivity is indicated as NeuAc titer per hour. All data were the average of three independent studies with standard deviations.

We next selected four different ribosome binding sites (RBSs) with different estimated translation-initiating rates[39] and five different inhibitory N-terminal coding sequences (NCSs) with different intensities[29] to regulate the expression of essential genes *folB* and *ftsW* in the translational level, to avoid the leakage (Figure 2a; Table S3). Then, we analyzed the growth of different strains in the absence or presence of 0.1 mM IPTG (Figure 2a,b) and found that the maximal OD_{600} of strains Nos. 12 (with essential gene *folB* regulated by sensor promoter P_{veg105} and RBS3) and 21 (with essential gene *ftsW* regulated by P_{veg105}, RBS0, and NCS4) with 0.1 mM IPTG was reduced by 36.2% and 30.3%, respectively, compared to those without IPTG, and recovered with the further addition of 2 g/L NeuAc. To confirm whether iPopQC coupled cell growth to NeuAc synthesis via controlling the expression of essential genes, RT-qPCR was conducted to analyze the expression levels of *folB* and *ftsW* of strains Nos. 12 and 21, respectively. As shown in the Figure S7, when iPopQC was initiated by adding 0.1 mM IPTG at 0 h of cultivation, the relative expression levels of *folB* and *ftsW* of strains Nos. 12 and 21 without NeuAc addition decreased to 58% and 65% of those in the absence of 0.1 mM IPTG, respectively. The relative expression levels of *folB* and *ftsW* of strains Nos. 12 and 21 with the addition of 0.1 mM IPTG and 2 g/L NeuAc were 1.64- and 1.96-fold, respectively, compared to those adding only 0.1 mM IPTG. In summary, these results indicate that iPopQC successfully coupled cell growth to NeuAc synthesis via regulating essential genes, conferring a growth advantage on the producing cell. In the previous study, a STAPL system was developed in *E. coli* to maintain plasmid stability and tune plasmid copy number by expressing essential gene *infA* on plasmid.[40] The STAPL system increased the plasmid copy number by a low expression level of essential gene *infA* on plasmid and did not influence cell growth.[40] This phenomenon can impair the coupling of the cell growth to NeuAc production. However, we did not observe the same phenomenon as the STAPL system in iPopQC, which may be due to the difference in essential genes and production host.

Validating iPopQC Systems for Improved NeuAc Synthesis. To test the performance of iPopQC in improving NeuAc synthesis, we introduced iPopQC systems successfully coupling NeuAc synthesis to cell growth into NeuAc-producing strain No. 1, respectively, resulting in strains Nos. 23 (with essential gene *folB* regulated by P_{veg105} and RBS3) and 24 (with essential gene *ftsW* regulated by P_{veg105}, RBS0, and NCS4). Then, we analyzed their NeuAc productions and found that when adding 0.1 mM IPTG at 0 h of cultivation to initiate iPopQC, 2.54 and 2.33 g/L NeuAc production was achieved, respectively, representing 1.30- and 1.22-fold increase compared to those without IPTG addition (1.96 and 1.91 g/L, respectively) and 1.18- and 1.08-fold NeuAc production of strain No. 1 without iPopQC (2.15 g/L) (Figure 3a,b). Strains Nos. 23 and 24 in the medium without IPTG addition produced slightly lower NeuAc than strain No. 1, which may be due to the following two reasons: (1) perturbation of intracellular essential metabolism and (2)

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protein production burden from expressing genetic components of iPopQC. To confirm this, we constructed strain No. 31 (No. 32) by controlling *gfp* using IPTG-inducible NeuAc-responsive biosensor in the transcriptional level and RBS3 (RBS0 and NCS4) in the translational level in strain No. 1. When IPTG was not added to the medium, NeuAc titers of strains Nos. 31 and 32 were reduced by 10% and 8.8%, respectively, compared to that of strain No. 1, showing almost the same values as those of strains Nos. 23 and 24, respectively (Figure S8), indicating that the protein production burden from expressing genetic components of iPopQC may be the main reason for the slightly lower NeuAc titer.

The previous PopQC (product addiction) functioned through the entire fermentation. However, when cell growth is affected by a low expression level of growth determinant genes, biosynthesis may be impaired, due to insufficient product synthesis during the early growth phase. In contrast, iPopQC can mitigate this by dynamic cell subpopulation regulation via changing the time of adding the inducer. To compare the effectiveness of PopQC and iPopQC, 0.1 mM of IPTG was added to the fermentation medium at different time points, including 0 h (the same function as PopQC), the lag (2.5 h), early logarithmic (5 h), mid logarithmic (7.5 h), and late logarithmic (10 h) phases during cultivation (Figure S9). We found that when IPTG is added to the medium at 2.5 and 5 h of cultivation, the NeuAc titers of strains Nos. 23 (3.86 g/L) and 24 (3.69 g/L) were 1.97- and 1.90-fold those of the controls without IPTG addition, and 1.52- and 1.59-fold those of the static cell subpopulation controls, respectively (Figure 3a,b). We then analyzed NeuAc conversion yields. When adding IPTG to the medium at 2.5 and 5 h of cultivation, the conversion yields of strains Nos. 23 and 24 were 2.09- and 1.9-fold higher than those of the controls without IPTG addition, and 1.38- and 1.41-fold higher than those of the static cell subpopulation controls, respectively (Figure S10). These results imply the importance of the dynamic control property of iPopQC.

Verifying Enriched Nongenetic Producing Cell Subpopulation by iPopQC. To test whether iPopQC improved bioproduction by enriching the producing cell subpopulation during cultivation, we compared the distribution of different cell subpopulations when coupling or not coupling cell growth to NeuAc synthesis. In the iPopQC systems, essential genes were downregulated at the translational level by RBS or NCS engineering to avoid the leakage expression of the sensor promoter. Therefore, it is likely that direct fusion of the *gfp* reporter and essential gene in the same cistron used in the previous study[17] cannot distinguish between the producing and nonproducing cell subpopulations, due to the low output signal of the sensor promoter. Therefore, we next coexpressed the *gfp* reporter gene and the essential gene in iPopQC in the form of polycistron (Figure 4a,b), to analyze the distribution of different cell subpopulations. To confirm whether the introduction of the *gfp* reporter gene affected the coupling of cell growth to NeuAc synthesis and NeuAc production, we first introduced the iPopQC coexpressing *gfp* and essential gene into the nonproducing strain No. 5 with the heterologous expression of NeuAc-transporter NanT, resulting in strains Nos. 33 (coexpressing *gfp* and *folB* regulated by RBS3) and 34 (coexpressing *gfp* and *ftsW* regulated by RBS0 and NCS4) (Figure 4a,b). We found that compared to those in the absence of 0.1 mM IPTG and NeuAc, strains Nos. 33 and 34 showed decreased growth with 0.1 mM IPTG addition, and growth recovery with the simultaneous addition of 0.1 mM IPTG and NeuAc (Figure S11). Furthermore, we introduced the iPopQC coexpressing *gfp* and essential gene into producing strain No. 1, resulting in strains Nos. 27 (coexpressing *gfp* and *folB* regulated by RBS3) and 28 (coexpressing *gfp* and *ftsW* regulated by RBS0 and NCS4) (Figure 4a,b), which displayed 1.37- and 1.36-fold increases in NeuAc titer when 0.1 mM of IPTG was added to the medium at 0 h of cultivation, compared to those without IPTG (Figure S12), respectively. Therefore, we confirmed that introduction of the *gfp* reporter gene did not affect the coupling of cell growth to NeuAc synthesis and NeuAc production. Next, we revealed the distribution of different cell subpopulations using flow cytometry when cell growth was coupled or not coupled to NeuAc synthesis (Figure S13). Taking the strains Nos. 25 and 26 without deleting genome copies of essential genes as uncoupling controls, we found that the proportions of producing cell subpopulations from strains Nos. 27 and 28 (coupling cell growth to NeuAc production) (Figure 4c) increased by 48.8% and 23.7% at 55 h of cultivation, respectively, compared to those not coupling cell growth to NeuAc synthesis, indicating that iPopQC improved bioproduction by enriching the producing cell subpopulation during cultivation.

Furthermore, we analyzed the productivities of strains Nos. 23 (coupling NeuAc synthesis to cell growth by regulating *folB* expression) and 24 (coupling NeuAc synthesis to cell growth by regulating *ftsW* expression) (Figure 4d). If iPopQC enriches the producing cell subpopulation during cultivation, the productivity should increase at multiple time periods especially in the late fermentation, compared to that of the control without initiated iPopQC (in the absence of IPTG). Indeed, the productivities of both strains with initiated iPopQC (in the presence of IPTG) increased in the late period of fermentation as shown in Figure 4d, compared to that of the control without initiated iPopQC (in the absence of IPTG). Therefore, the results indicate that iPopQC improved bioproduction by enriching the producing cell subpopulation during cultivation.

Genetic variation and nongenetic variation can both result in bioproduction heterogeneity. To illustrate whether mutation caused heterogeneity for NeuAc production in our study, we isolated offspring colonies from cultures of strain No. 23 (with IPTG inducible population quality control system) treated with or without IPTG for population quality control or not, respectively. The colonies were further used for testing NeuAc production. If nongenetic variation results in bioproduction heterogeneity, colonies from strain No. 23 treated with IPTG should have similar NeuAc titers in the absence and presence of IPTG (added at 2.5 h of cultivation), compared to those of strain No. 23 in the absence and presence of IPTG (added at 2.5 h of cultivation), respectively. In addition, colonies from strain No. 23 treated without IPTG should also have similar NeuAc titers in the absence and presence of IPTG (added at 2.5 h of cultivation), compared to those of strain No. 23 in the absence and presence of IPTG (added at 2.5 h of cultivation), respectively. When recultivating, colonies from strain No. 23 treated with IPTG indeed showed similar NeuAc titers (1.92 g/L and 3.90 g/L) in the absence and presence of IPTG (added at 2.5 h of cultivation), compared to those of strain No. 23 (1.96 g/L and 3.86 g/L), respectively (Figure S14). Recultivations of colonies from strain No. 23 treated without IPTG also showed the same results (1.88 g/L and 3.87 g/L).

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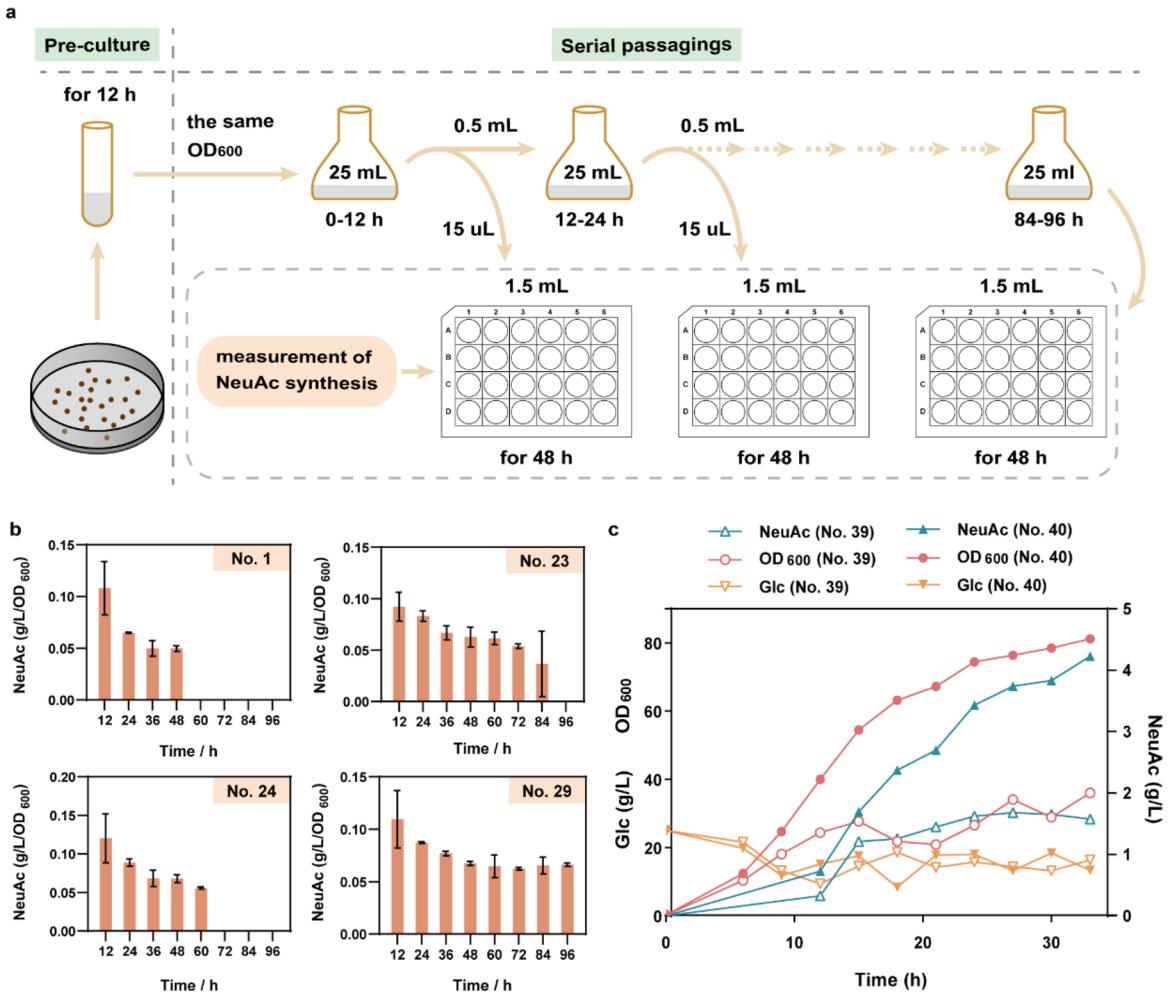

Figure 5. Improved production robustness by iPopQC. (a) Serial passaging to simulate experimental duration of the scale-up of the bioprocess for analyzing production robustness. Starting from single colonies and then seed cultures, shaking flask cultures were passaged serially every 12 h for a total of eight times. At the time of passaging, the cultures were simultaneously inoculated into 24-well deep plates with 1.5 mL of fresh fermentation medium at an inoculum rate of 2% and then cultivated at 37 °C and 220 rpm for 48 h for measurement of NeuAc synthesis. (b) NeuAc production for long-term cultivated strains Nos. 23, 24, 29, and 1. Cells were serial passaged according to the workflow depicted in panel a. Cultures of 48 h in 24-well deep plates were collected for analyzing NeuAc production capacity and measurement of OD_{600}. All data were the average of three independent studies with standard deviations. (c) NeuAc production of strains No. 39 (without iPopQC) and 40 (with single-output iPopQC) in a 3 L fermenter.

Therefore, the improved production phenotype by iPopQC is nongenetic. In addition, six colonies from each culture with or without IPTG addition for population quality control were selected for whole genome sequencing (Supplementary Note). Compared to the genome sequence of the parent strain No. 23, no mutations were found on any components of iPopQC construct and NeuAc synthetic pathway in all colonies from each culture (with or without IPTG) (Supplementary Note). Therefore, nongenetic variation caused the heterogeneity in NeuAc production.

Improved Production Robustness by iPopQC. In iPopQC, genetic mutations in the sensor promoter may cause continuous expression of the cell growth determinant genes, invalidating population quality control systems. To avoid this, we designed double-output iPopQC (Figure 1b) with the sensor promoter separately controlling two cell growth determinant genes in two expression cassettes. Double-output iPopQC is expected to confer improved robustness to iPopQC by reducing the possibility of iPopQC dysfunction, which only occurs by introducing mutations in both sensor promoters, therefore improving NeuAc-producing robustness further. Therefore, we constructed strain No. 29 with double-output iPopQC, which was derived from strain No. 1 by controlling both *folB* (simultaneously regulated by RBS3 in the translational level) and *ftsW* (simultaneously regulated by RBS0 and NCS4 in the translational level) using IPTG-inducible NeuAc-responsive biosensor in the transcriptional level in two expression cassettes (Figure S15), followed by knocking out the original genome copies of *folB* and *ftsW* genes. To confirm whether double-output iPopQC further improved production robustness, the three strains Nos. 23 (with single-output iPopQC), 24 (with single-output iPopQC), and 29 (with double-output iPopQC) carrying iPopQC and strain No. 1 without iPopQC were passaged serially to experimentally simulate the duration of scaling-up of the bioprocess[18,41,42] (Figure 5a). We passaged the four strains every 12 h for a total of eight times. At the time of passaging, 0.1 mM of IPTG was added to the medium to functionalize iPopQC. We found that the production robustness of strains Nos. 23 and 24 with single-output iPopQC were improved by

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75% and 25%, in comparison with strain No. 1 without iPopQC (Figure 5b). The two strains Nos. 23 and 24 both retained NeuAc production for at least 60 h of cultivation. In contrast, the control strain No. 1 completely lost the NeuAc-producing ability at 60 h of cultivation. Moreover, strain No. 29 with double-output iPopQC showed improved robustness compared to the two strains Nos. 23 and 24 with single-output iPopQC, and still could retain NeuAc production throughout 96 h of fermentation, at which point the two strains Nos. 23 and 24 completely lost the NeuAc-producing ability. These results imply that double-output iPopQC is effective for maintaining the target metabolite-producing ability, which displayed greater application potential for long-term industrial biosynthesis.

To test the robustness of an iPopQC at larger scale, fermentation of the NeuAc-producing strain was tested in a 3 L fermenter. We first expressed NeuAc pathway genes *age* and *neuB* from plasmid pB6CG under the control of different promoters (P_{odh}, P_{S66}, and P_{yvy}) on a pHT01 vector in strain No. 2, generating strains Nos. 37, 38, and 39, respectively, and then analyzed their performances in NeuAc synthesis. Strain No. 39 with *age* and *neuB* regulated by P_{yvy} has the highest NeuAc production, reaching 2.27 g/L (the strain No. 1 produced 2.15 g/L NeuAc) in 24-well deep plates after 60 h of cultivation (Figure S16a). Next, the strain No. 40 was constructed by introducing iPopQC coupling synthesis to cell growth by engineering *folB* into strain No. 39. Subsequently, we tested the performance of strains Nos. 39 (without iPopQC) and 40 (with single-output iPopQC) in a 3 L fermenter (Figure 5c). Strain No. 40 achieved 2.5-fold increase in NeuAc titer compared to strain No. 39 (1.69 g/L), reaching 4.23 g/L. In addition, strain No. 40 achieved 1.77-fold increase in NeuAc conversion yield (0.039 g/g glucose) compared to strain No. 39 (0.022 g/g glucose). Those results indicate that iPopQC can effectively maintain the target metabolite-producing ability at larger scale.

Overall, we developed iPopQC to dynamically modulate the producing and nonproducing subpopulations of engineered *B. subtilis* during fermentation. There are three major advantages of iPopQC compared to previous population quality control systems. First, the inducibility of iPopQC expanded the range of applications for population quality control systems, enabling its suitability for both kinds of metabolites that can or cannot be transported into the cells. Second, in previous population quality control systems, insufficiency in product synthesis during the early growth phase may affect cell growth, which would further impair biosynthesis. In contrast, the inducibility of iPopQC avoided this detrimental impact by dynamic population quality control. Third, double-output iPopQC with improved robustness showed greater potential for long-term fermentation.

Population quality control systems require a product-responsive biosensor for which its operational range (i.e., range of product concentration over which the output signal exhibits significant changes[43]) matches the intracellular concentrations in the biosynthetic cell[18] and has broad dynamic range (i.e., fold-change in output signal between in the presence and absence of inducer). Therefore, developing tools that can tune the operational and dynamic ranges of a product-responsive biosensor efficiently would facilitate application of population quality control systems further. As for this, optimizing gene expression regulatory elements based on machine-learning is a promising venture.[44] In addition, IPTG needs to be added in iPopQC, causing increased cost. One possible solution to reduce inducer costs is to use a cheaper inducer, such as light. Recently, various optogenetic gene expression control systems have been developed, including red-, blue-, and green-light responsive systems, which lay the foundations for optogenetic control.[45,46] In addition, light as an inducer is not only inexpensive and nontoxic, but can also be instantaneously applied or removed,[45−47] due to which programmable population quality control is promising. When the expression of the components of iPopQC causes a heavy metabolic burden on the producing strain, pulsed PopQC induced by light pulses may be a promising solution.

In conclusion, iPopQC was developed for enriching the producing cell subpopulation of engineered B. subtilis during fermentation and achieved a 1.97-fold increase in the NeuAc titer. In addition, compared to previous population quality control systems, iPopQC not only broadened the applicability and robustness of previous population quality control systems, but also improved the biosynthesis efficiency further by dynamically controlling population quality. In terms of the prevalence of cell-to-cell variation and development of various metabolite-responsive biosensors, iPopQC may be broadly applicable and effective for improving the biosynthesis robustness and efficiency of various important biomolecules.

METHODS

Plasmids, Strains, and Culture Conditions. All plasmids used in this study are listed in Table S1. The plasmids used for building iPopQC were constructed with Gibson assembly based on vector plasmid pHT01[48,49] or by substituting RBS of the essential gene or inserting NCS upstream of the essential gene in the constructed plasmid using modified primers. They were then extracted from *Escherichia coli* JM109 that was used for cloning purposes and transported into *B. subtilis* to construct the strains listed in Table S2. The *Cre/lox* system and PCR-based genome engineering in *B. subtilis* were used to knock out genome copies of essential genes.[50] All transformations into *B. subtilis* were conducted according to the previously described method.[51] When preparing competent cells and seed cultures, all strains were cultured in Luria−Bertani (LB) broth and agar plates with appropriate antibiotics (100 mg/L ampicillin for *E. coli*, 50 mg/L kanamycin, and/or 5 mg/L chloramphenicol for *B. subtilis*) at 220 rpm and 37 °C, unless otherwise stated. The fermentation medium used for NeuAc production in 24-well deep plates, growth monitoring in 24-well deep plates, and seed culture used for the fed-batch fermentation in a 3-L fermenter consisted of yeast extract, 12 g/L; peptone, 6 g/L; $(NH_4)_2SO_4$, 6 g/L; $K_2HPO_4{\cdot}4H_2O$, 12.5 g/L; KH_2PO_4, 2.5 g/L; $MgSO_4{\cdot}7H_2O$, 3 g/L; urea, 6 g/L; and glucose, 60 g/L (pH 7.0).[26] When needed, 0.1 mM of IPTG or 2 g/L of NeuAc (2 g/L was enough to activate the NeuAc-responsive biosensor and closed to the NeuAc production of strain No. 1) was added to the fermentation medium at 0 h of cultivation unless otherwise stated.

Measurements of OD_{600}, GFP Fluorescence, and NeuAc Production. A single colony cultured on an LB agar plate was inoculated in 24-well deep plates with 1.5 mL of fermentation medium, and cells were cultivated at 37 °C and 220 rpm for a specific time. A 200 μL portion of culture was directly used for measurement of GFP fluorescence by a microplate Multi-Mode Reader (BIOTEK, Cytation 3) at an excitation wavelength of 488 nm and emission wavelength of

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523 nm with a gain value of 60. To ensure consistency, FL/OD_{600} was used to normalize GFP fluorescence. For the measurement of cell growth, which was represented by the OD_{600} value, the culture was diluted to OD_{600} = 0.1−0.8 and transferred into 96-well microwell plates. Then, the OD_{600} value of the 200 μL diluted culture was measured using a microplate Multi-Mode Reader (BioTek, Cytation 3). To remove the background, the OD_{600} value was computed by subtracting the OD_{600} value of the fermentation medium diluted with the same folds as the culture. For quantification of NeuAc production, culture was harvested and then centrifuged at 14 000 rpm for 10 min. Then, 300 μL of the supernatant was transferred to 600 μL of 35 mM H_2SO_4 to remove soluble proteins. After centrifugation at 14 000 rpm for 10 min, the supernatant was filtered with 0.22-μm filter and then used to measure the concentrations of NeuAc and glucose (Glc) by HPLC.

Flow Cytometry Analysis. Cells that needed analyzing were cultured as described above then collected and washed with filtered PBS buffer, after which the cells were suspended in filtered PBS buffer with an OD_{600} of 0.3−0.5. Treated cells were kept on ice until use. Prior to flow cytometry analyses, samples were vortexed thoroughly. The analysis was performed using a BD FACSArica III with an excitation source of 488 nm and an emission window of 523/21 nm. Approximately 10000−20000 events were analyzed for each sample. Forward scatter and side scatter were in logarithmic amplification and set to exclude background events. Data were analyzed and visualized using FlowJo software.

Mathematical Simulation. Matlab 2019b was used to build the model. The growth curve data of the strains in the model are obtained by interpolation based on the experimental results of the growth curve of *B. subtilis* No. 4. For the simulation of fermentation in the presence of the nonproducing cell subpopulation, the proportion of the nonproducing cell subpopulation is calculated according to the flow cytometry results (Figure 1a): I_Nonproducing (%) = $1.0729t + 17.833$, $R^2 = 1$ (t is the fermentation time). Since the NeuAc biosensor can only be used to analyze the distribution of the producing cell subpopulation and nonproducing cell subpopulation in NeuAc synthesis performance, the NeuAc produced by the producing cell subpopulation per unit time unit biomass in the model is set to 1 (arbitrary unit, au). Thus, NeuAc yield (au) is the sum of NeuAc produced by the producing cell subpopulation at all times. For the simulation of fermentation in the absence of the nonproducing cell subpopulation, NeuAc yield (au) is the sum of NeuAc produced by all cells at all times.

Construction and Characterization of iPopQC. The P_{grac2} fragment including lacI was amplified from pHT100[52,53] via primer pairs pCGSV-1F/pCGSV-1R. *nanR* and P_{veg105}-gfp fragments were amplified from plasmid veg105-nanR6K using primer pairs pCGSV-2F/pCGSV-2R and pCGSV-3F/pCGSV-3R, respectively. Then the three fragments were assembled with linearized veg105-nanR6K by PCR via primer pairs pCGSV-4F/pCGSV-4R, generating plasmid pCGSV, on which the transcriptional factor NanR was under the control of promoter P_{grac2}. Then, the plasmid was introduced into the nonproducing strain No. 2 and NeuAc-producing strain No. 1, generating strains Nos. 3 and 4. To confirm the optimal IPTG concentration, single colonies of the two strains cultured on LB agar plates were inoculated in 1.5 mL of fermentation medium with different concentrations of IPTG and proper antibiotics. Then, cultures were harvested after 24 h of cultivation for the measurements of GFP fluorescence and OD_{600} to confirm the optimal concentration of IPTG, at which the NeuAc-responsive biosensor had a maximal fold-change between in the absence and presence of NeuAc.

Four essential genes, which were selected based on the subtiwiki database,[34] were amplified from *B. subtilis* genome using primer pairs in Table S3, and then were used to replace the *gfp* gene in plasmid pCGSV, generating plasmids pCGSA, pCGSD, pCGSF, and pCGSFt, respectively. To avoid leakage from the biosensor, we substituted RBS or inserted inhibitory NCS upstream of the essential genes in pCGSF and pCGSFt using modified primers (Table S3). To construct double-output iPopQC, the P_{veg105}-RBS0-NCS4-ftsW fragment was amplified from plasmid pCGSFtN4 by PCR via primer pairs FtN4-F/FtN4-R, and then was ligated with linearized pCGSF3 by PCR via primer pairs F3-F/F3-R. This results in plasmid pCGS-F3FtN4, on which the *folB* gene is under the regulation of P_{veg105} and RBS3, and the *ftsW* gene is under the regulation of P_{veg105}, RBS0, and NCS4 in the form of two monocistrons. To test whether iPopQC improved bioproduction by enriching the producing cell subpopulation during cultivation, the *folB* fragment was amplified from pCGSF3 using primer pairs GF3-F/GF3-R, and then was assembled with linearized pCGSV by PCR via primer pairs L2-pCGSV-F/L2-pCGSV-R, generating plasmid pCGS-GF3. The *ftsW* fragment was amplified from pCGSFtN4 using primer pairs GFtN4-F/GFtN4-R, and then was assembled with linearized pCGSFtN4 by PCR via primer pairs L- pCGSFtN4-F/L-pCGSFtN4-R, generating plasmid pCGS-GFtN4.

These plasmids were introduced into strain No. 5, and the genome copy of the corresponding essential gene was knocked out using *the Cre/lox* recombination system and PCR-based genome engineering in *B. subtilis*.[49] To confirm whether cell growth was coupled to synthesis, single colonies of the strains cultured on LB agar plates were inoculated in 24-well deep plates with 1.5 mL of fermentation medium under different circumstances of adding IPTG, not adding IPTG, or adding IPTG and NeuAc simultaneously. Cells were cultivated at 37 °C and 220 rpm and cultures were harvested for measurement of OD_{600}. To test the performance of iPopQC in improving NeuAc synthesis and enriching the producing cell subpopulation, iPopQC plasmids that successfully coupled cell growth to NeuAc synthesis were introduced to strain No. 1, followed by deleting genome copies of corresponding essential genes. Then single colonies cultured on LB agar plates were inoculated in 1.5 mL of fermentation medium with or without 0.1 mM of IPTG and were cultivated at 37 °C and 220 rpm. Cultures were next harvested for NeuAc synthesis after 60 h of cultivation and for flow cytometry analysis after being cultivated for a specific time.

RT-qPCR Analysis. To analyze the relative expression level of the essential genes in the iPopQC in transcriptional level, single colonies of strains Nos. 12 and 21 cultured on LB agar plates were inoculated in 1.5 mL of fermentation medium in a 24-well plate with adding IPTG, not adding IPTG, and adding IPTG with NeuAc simultaneously. The colonies were cultivated at 37 °C and 220 rpm for 20 h, and then 0.5 mL of the cultures were then harvested for RNA extraction. RNA extraction was performed according to the standard operation manuals of RNAprep Pure Cell/Bacteria Kit (Tiangen Biotech Company, Beijing). The quantity and purity of RNA were determined by optical density measurements at 260 and 280

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nm using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Reverse transcription was conducted with PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Biomed Company, Dalian) to obtain cDNA. With the cDNA as template, qPCR was performed in a 96-well plate with a total reaction volume of approximately 20 μL using TB Green Premix Ex *Taq*II (Tli RNaseH Plus) (Takara Biomed Company, Dalian). The reactions were conducted with a LightCycler480 II System (Roche Applied Science, Mannheim, Germany) based on the standard operation manual of TB Green Premix Ex *Taq*II (Tli RNaseH Plus). The *hbsU* gene was used as the internal standard.[54]

Test of Strain Production Robustness. To test production robustness of the strains, single colonies cultured on LB agar plates were inoculated into LB liquid for 12 h at 37 °C and 220 rpm to prepare seed liquids. The seed liquids were transferred to 25 mL of fermentation medium in a 250 mL shaking flask at an inoculum rate of 2% and the same initial OD_{600} value. After 12 h of cultivation at 37 °C and 220 rpm, the cultures were passed to 25 mL of fresh fermentation medium in a 250 mL shaking flask at an inoculum rate of 2% and the passaged cells were cultivated under the same conditions for another 12 h, which was then repeated seven times. At the time of passaging, the cultures were simultaneously inoculated into 24-well deep plates with 1.5 mL of fresh fermentation medium at an inoculum rate of 2% for NeuAc production. Cultures were harvested after 48 h of cultivation for measurement of NeuAc titer.

Moreover, for fed-batch fermentation in a 3-L fermenter, the medium was composed of 20 g/L yeast extract, 20 g/L tryptone, 12 g/L $K_2HPO_4{\cdot}4H_2O$, 2.5 g/L KH_2PO_4, 2.5 g/L $MgSO_4{\cdot}7H_2O$, 10 g/L urea, and 25 g/L initial glucose. First, colonies in the LB agar plates were inoculated into 5 mL of LB medium and cultured at 37 °C and 220 rpm for 7 h. Then the LB cultures were inoculated into two 250 mL shake flasks containing 50 mL of fermentation medium at an inoculum rate of approximate 5%, which were then cultured at 37 °C and 220 rpm for 7 h (OD_{600} = 5−6). Next, 100 mL of the culture in the fermentation medium was inoculated into a 3 L fermenter (T&J-Atype; T&J Bioengineering Co., Ltd., Shanghai, China) with an initial 1.8 L of fermentation medium. The pH was kept at 7.2 using 30% NH_4HO, the temperature was maintained at 37 °C, and the aeration rate was maintained at 1.5 vvm. In addition, the agitation speed was maintained at 500 rpm for the first 6 h of cultivation and 750 rpm after 6 h of cultivation. Glucose concentration was maintained within 10−25 g/L by feeding with 600 g/L glucose, and the feeding rates were adjusted based on the change of glucose concentrations in the fermentation medium.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00086.

Schematic diagram of engineering the four essential genes, relative transcriptional expression levels of *folB* and *ftsW* genes, NeuAc conversion yields, and cell subpopulation distribution of strains with iPopQC and so on; detailed information on plasmids, strains, and primers; experimental procedures on genome sequencing and detailed information on nonsynonymous SNPs (Nonsyn) and indel mutations (Indel) (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Guocheng Du − *Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education and Science Center for Future Foods, Jiangnan University, Wuxi 214122, China*; Phone: +86-510-85918309; Email: gcdu@jiangnan.edu.cn; Fax: +86-510-85918309

Yanfeng Liu − *Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education and Science Center for Future Foods, Jiangnan University, Wuxi 214122, China*; orcid.org/0000-0002-0562-9647; Phone: +86-510-85197117; Email: yanfengliu@jiangnan.edu.cn; Fax: +86-510-85918309

Authors

Yanting Cao − *Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education and Science Center for Future Foods, Jiangnan University, Wuxi 214122, China*

Rongzhen Tian − *Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education and Science Center for Future Foods, Jiangnan University, Wuxi 214122, China*

Xueqin Lv − *Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education and Science Center for Future Foods, Jiangnan University, Wuxi 214122, China*

Jianghua Li − *Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education and Science Center for Future Foods, Jiangnan University, Wuxi 214122, China*

Long Liu − *Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education and Science Center for Future Foods, Jiangnan University, Wuxi 214122, China*

Jian Chen − *Science Center for Future Foods and National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.1c00086

Author Contributions

Y.C. and Y.L. designed the experiments. Y.C. and R.T. performed the biochemical experiments and analyzed the data. X.L., J.L., L.L., G.D., J.C., and Y.L. conceived the project and supervised the research. Y.C., R.T., X.L., J.L., L.L., G.D., J.C., and Y.L. wrote the manuscript.

Notes

The authors declare no competing financial interest.

Data availability. All data discussed in this study can be found in this article (and its Supporting Information files).

Code availability. The MATLAB code used in this study can be found in GitHub [https://github.com/Tian-RZ/NeuAc_Non_producing_cell_subpopulation.git].

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