Improvement of putrescine production through the arginine decarboxylase pathway in Escherichia coli K-12

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Abstract
In the bio-based polymer industry, putrescine is in the spotlight for use as a material. We constructed strains of Escherichia coli to assess its putrescine production capabilities through the arginine decarboxylase pathway in batch fermentation. N-Acetylglutamate (ArgA) synthase is subjected to feedback inhibition by arginine. Therefore, the 19th amino acid residue, Tyr, of argA was substituted with Cys to desensitize the feedback inhibition of arginine, resulting in improved putrescine production. The inefficient initiation codon GTG of argA was substituted with the effective ATG codon, but its replacement did not affect putrescine production. The essential genes for the putrescine production pathway, speA and speB, were cloned into the same plasmid with argAATG Y19C to form an operon. These genes were introduced under different promoters; lacI, lacIq, lacIq1p, and T5p. Among these, the T5 promoter demonstrated the best putrescine production. In addition, disruption of the puuA gene encoding enzyme of the first step of putrescine degradation pathway increased the putrescine production. Of note, putrescine production was not affected by the disruption of patA, which encodes putrescine aminotransferase, the initial enzyme of another putrescine utilization pathway. We also report that the strain KT160, which has a genomic mutation of YifEQ100TAG, had the greatest putrescine production. At 48 h of batch fermentation, strain KT160 grown in terrific broth with 0.01 mM IPTG produced 19.8 mM of putrescine.

Keywords: Glutamate-putrescine ligase, N-acetylglutamate synthase, Macrodomain Ori protein, Terrific broth

Introduction
Putrescine is a polyamine and consists of two amino groups and four methylene groups. This compound is widely distributed in living organisms (Pegg 1986; Michael 2016; Keller et al. 2019). Several studies have focused on putrescine as it regulates rapid cell proliferation and differentiation at the level of gene expression (Heby 1981; Michael 2018; Igarashi and Kashiwagi 2000, 2018, 2019). Yoshida et al. (2004) reported a correlation between putrescine and more than 600 genes related to the regulation of transcription. In addition, putrescine is used as a precursor of other polyamines, surfactants, and agrochemicals, and as a component of polymers such as nylon 4,6 (Schneider and Wendisch 2011; Wendisch et al. 2018; Hui et al. 2020). The polycondensation of putrescine and adipic acid is used to synthesize nylon 4,6. Due to its flexibility and high solvent resistance properties, nylon 4,6 was introduced into the commercial field (Demco et al. 2007; Yamanobe et al. 2007). Polymides are used in the electric vehicle industry as materials to develop a lighter body, interior, motor, controller, and electronic board of the car. Along with the expansion of the global electric vehicle market, the demand for putrescine is also increasing (Scott et al. 2007; Schneider and Wendisch 2010). There are two processes to synthesize putrescine, chemical and biological. However, the chemical process producing an intermediate, succinonitrile, releases dangerous and harmful compounds.

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On the other hand, the biological process is safe, environmentally friendly, and uses renewable feedstock (Sanders et al. 2007; Nguyen and Lee 2019; Li et al. 2020a).

Among the living organisms that can produce putrescine, bacteria are commonly used in research because they grow rapidly under simple culture conditions such as Escherichia coli. The knowledge of polyamines in E. coli was reviewed admirably by Tabor and Tabor (1985) a long time ago, but it is still valid today. E. coli has two putrescine synthetic pathways (Fig. 1): the ornithine decarboxylase (ODC) pathway and the arginine decarboxylase (ADC) pathway, which use ornithine and arginine as starting compounds, respectively (Fig. 1). Arginine is synthesized from glutamate via ornithine decarboxylase (SpeC) and/or inducible ornithine decarboxylase (SpeF) (Tabor and Tabor 1985). The other pathway, the ADC pathway, converts arginine to putrescine via agmatine. The conversion of arginine to agmatine is catalyzed by biosynthetic arginine decarboxylase and putrescine via agmatine. The conversion of arginine to putrescine is catalyzed by biosynthetic arginine decarboxylase (SpeC) and/or inducible ornithine decarboxylase (SpeF) (Tabor and Tabor 1985). The other pathway, the ADC pathway, converts arginine to putrescine via agmatine. The conversion of arginine to agmatine is catalyzed by biosynthetic arginine decarboxylase (SpeA) and biodegradative arginine decarboxylase (AdiA). Subsequently, agmatine is hydrolyzed to putrescine by agmatinase (SpeB). Putrescine is catabolized by two pathways, the putrescine utilization pathway (Puu pathway) (Kurihara et al. 2005) and the putrescine aminotransferase (PatA)-γ-aminobutyraldehyde dehydrogenase (PatD) pathway. These catabolic pathways rely on glutamate-putrescine ligase (PuuA) and putrescine aminotransferase (PatA), respectively (Samsonova et al. 2003, 2005; Kurihara et al. 2008). S-Adenosylmethionine decarboxylase (SpeD) generates decarboxylated S-adenosyl-L-methionine. Its n-propylamine is transferred to putrescine via spermidine synthase (SpeE) to synthesize spermidine (Tabor et al. 1986). Spermidine N-acetyltransferase (SpeG) converts spermidine to its inactive form, N-acetyl spermidine (Limswun and Jones, 2000). In addition, Haywood and Large (1985) reported that speG encodes a putative diamine acetyltransferase that converts putrescine to acetylputrescine in Candida boidinii. Although the use of putrescine by E. coli SpeG as a substrate has not been reported, the speG gene was knocked out in this study to prevent the possible loss of putrescine (Fig. 1). Because of the cationic function of polyamine, it cannot penetrate through the cell membrane. Two spermidine transporters, PotABC and MdtI, and six putrescine transporters, PpuP, YdcSTUV, PpaP, PotE, PotFGHI, and SapBCDF, have been identified (Kashiwagi et al. 1992; Pistocchi et al. 1993; Kurihara et al. 2009a, b; Kurihara et al. 2011; Saier et al. 2016; Sugiyma et al. 2016). We recently reported that PotFGHI can import spermidine under biofilm-forming conditions (Thongbhubate et al. 2021).

Qian et al. (2009) overproduced putrescine through the ODC pathway in E. coli K-12, and the batch culture experiment resulted in 1.68 g/L (19.0 mM) in the 10 g/L of the glucose-enriched medium. Recently, Li et al. (2020b) overexpressed the ADC pathway and improved putrescine production using E. coli BL21(DE3). They produced putrescine using an SOB medium supplemented with 8 g/L of glucose and 12 g/L of arginine, resulting in 4.77 g/L of putrescine in batch culture. However, the latter method is a type of sequential enzymatic conversion of arginine to putrescine.

We found that the introduction of the plasmid that contains speAB produced more extracellular putrescine than that of the plasmids that contain speC or speABC. Therefore, we focused on fermentation of putrescine via the ADC pathway of E. coli K-12 without the addition of arginine. Here, genetic engineering was used as a tool to increase the production of putrescine. ArgA catalyzes the initial step of arginine biosynthesis (Rajagopal et al. 1998; Shin and Lee 2014; Ginesy et al. 2015). When arginine is overproduced, this compound affects N-acetylglutamate synthase (ArgA) activity through negative feedback inhibition. Furthermore, the DNA-binding transcriptional dual regulator (ArgR) represses the transcription of the genes of arg regulons, including argA. We not only attempted to enhance arginine synthesis, but also deleted genes related to the degradation or utilization of putrescine, in addition to transporters that uptake putrescine from the medium, such as potE, speG, patA, speD, argR and puuPA, and altered the expression of genes related to the synthesis of putrescine, speA, speB, and other genes from plasmids (Fig. 1).

Materials and methods

Construction of E. coli strains

The E. coli strains used in this study are listed in Table 1. The chromosomal genes speD, and argR were replaced with FRT (FLP recombination target)-kanR-FRT from the Keio gene knockout collection (Baba et al. 2006) by P1vir transduction (Miller 1972). The disruption of speG, potE, and the ATP binding site of the puuPA gene were described previously (Kurihara et al. 2008, 2009b; Thongbhubate et al. 2021). The patA and potFGHI genes were disrupted by a method described previously (Datsenko and Wanner 2000) using pKD13 as a template for the PCR amplification with delta-ygjG F and delta-ygjG R as primers for patA, and potF-up and potl-down as primers for potFGHI (Additional file 1: Table S1). The kanR genes were eliminated from FRT-kanR-FRT by Flp flippase carried by pCP20 (Datsenko and Wanner 2000). To confirm the deletion of genes, colony PCR was performed with primers (Additional file 1: Table S1) that annealed upstream and downstream of the genomic regions of.
Fig. 1 The metabolic map of putrescine in *E. coli* K-12. The Xs indicate the knocked out genes. Thick arrows indicate the overexpression of genes. Dash arrows indicate the increased expression of genes. Blunt end arrows indicate inhibition of the expression of genes. GdhA glutamate dehydrogenase, HdfR DNA-binding transcriptional dual regulator HdfR, GltBD glutamate synthase, GlnA glutamine synthetase, GlnE glutamine synthetase adenylyltransferase, CarAB carbamoyl phosphate synthetase, ArgA N-acetylglutamate synthase, ArgB acetylglutamate kinase, ArgC N-acetylglutamatephosphate reductase, ArgD N-acetylornithine aminotransferase, ArgF ornithine carbamoyltransferase, ArgI ornithine carbamoyltransferase, ArgR DNA-binding transcriptional dual regulator ArgR, SpeA biosynthetic arginine decarboxylase, SpeB agmatinase, SpeC constitutive ornithine decarboxylase, SpeD S-adenosylmethionine decarboxylase, SpeE spermidine synthase, SpeF inducible ornithine decarboxylase, SpeG spermidine N-acetyltransferase, PatA putrescine aminotransferase, PatD γ-aminobutyraldehyde dehydrogenase, GabaT 4-aminobutyrate aminotransferase, Sad succinate-semialdehyde dehydrogenase GabD, Sad succinate-semialdehyde dehydrogenase Sad, PuuB γ-glutamylputrescine ligase, PuuC γ-glutamyl-γ-aminobutyraldehyde, PuuD γ-glutamyl-γ-aminobutyrate hydrolase, PuuP putrescine importer, YdcSTUV putrescine importer, PatFGHI putrescine importer, PotFGHI putrescine importer, PotE putrescine-ornithine antiporter/putrescine importer, SapB putrescine exporter, TCA cycle tricarboxylic acid cycle.
the target genes, and the sizes of the amplicons were measured. To construct SH2204, the \( \text{yifE}^{-}\text{FRT-kan}\) of SH2201 was replaced with the \( \text{yifE}^{\text{Q100TAG}}\) using the \( \text{zie-296}\) phage grown on SH2203 to obtain SH2204, which was confirmed to be \( \text{Kan}^{\text{R}}\). Then, the \( \text{yifE} \) region of SH2204 was amplified by PCR and the DNA sequence was confirmed. For DNA modification and strain construction, \( \text{E. coli} \) cells were grown in LB medium (Miller 1972). Where appropriate, culture media were supplemented with 30 \( \mu \text{g/mL} \) of kanamycin, 100 \( \mu \text{g/mL} \) of ampicillin, or 15 \( \mu \text{g/mL} \) of tetracycline. For the pre-cultures, 10 \( \text{mL} \) of LB medium was inoculated with a single colony in 100- \( \text{mL} \) Erlenmeyer flasks. Cultures were incubated at 37 °C with reciprocal shaking at 120 rpm.

### Plasmid construction

The plasmids used in this study are shown in Table 2 and the primers used to construct the plasmids are listed in Additional file 1: Table S2. The Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, WI) was used to extract plasmid DNA from cells. PCR amplification was performed using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The QuikChange method (Stratagene; San Diego, CA) was used to generate \( \text{argA} \) of pSH1733 using KOD-plus DNA polymerase. There is a 137-base pair space between the \( \text{speA} \) and \( \text{speB} \) genes in the genome of MG1655. Therefore, the wild-type \( \text{speAB} \) genes may not be an operon. The \( \text{speA} \) and \( \text{speB} \) genes on the plasmids used in this study are different from the wild-type genes on the genome. They are designed to have only a 21-base pair space with a Shine Dalgarno (SD) sequence between them, i.e., they were designed to be an operon. Consequently, the transcription of \( \text{speA} \) will also pass through \( \text{speB} \). The \( \text{speC} \) gene was amplified using genomic DNA of MG1655 as a template and primers with EcoRI and SacI recognition sites. After the amplified fragment was cleaved with these two enzymes, it was inserted between the EcoRI and SacI sites of pQE-80L vector.

### Medium and culture conditions for putrescine production

Pre-culture was carried out in 10 \( \text{mL} \) of LB medium in a 100- \( \text{mL} \) Erlenmeyer flask for 16 h at 37 °C with a reciprocal shaking at 120 rpm. After 16 h, the pre-culture was transferred to 10 \( \text{mL} \) of LB or terrific broth medium to adjust the initial density of the cells to an \( \text{OD}_{600} \) of 0.05. The cultures were incubated at 37 °C with a reciprocal shaking at 120 rpm until the turbidity \( \text{OD}_{600} \) reached 0.4. Then, IPTG was added to the main culture as needed. When ampicillin was required, 100 \( \mu \text{g/mL} \) of ampicillin was added to the media.
Sample preparation
The culture was collected at the indicated time and used to measure the turbidity at OD$_{600}$. The collected cultured was centrifuged at 18,000 × g at 4 °C for 5 min. The supernatants were collected, and 25 μL of 100% (w/v) trichloroacetic acid was mixed with 250 μL of the three supernatants. Samples were filtrated through a Millex-LH Syringe Driven Filter Unit (Millipore; Billerica, MA).

Measurement of putrescine concentrations
The concentration of putrescine was measured using an LC-20 HPLC device (Shimadzu; Kyoto, Japan) equipped with a TSKgel Polyaminepak (Tosoh; Tokyo, Japan), as described previously (Kurihara et al. 2008). The temperature of the column was set at 50 °C and only one mobile phase solution was used. The flow rate of the mobile phase solution was 0.5 mL/min and that of the detection reagent was 0.42 mL/min. The peak of putrescine was detected at 6 min under this analytical condition. Putrescine was purchased from Nacalai Tesque (Kyoto, Japan). The amounts of polyamines are presented as the average of three independent cultures with standard deviations.

Table 2
| Strains | Genotype | Source or reference |
|---------|----------|---------------------|
| pCP20   | pSC101 replicon (Ts) bla$^+$ cat$^+$ | Datsenko and Wanner 2000 |
| pFS29   | pQE-80L except Tsp lacO_lacO_ speC$^+$ | This study |
| pKD13   | oriRb bla$^+$ FRT-Kan$^R$-FRT | Datsenko and Wanner 2000 |
| pKN11   | pQE-80L except Tsp lacO_lacO_ speA_speB_argA$^{ATG,Y19C}$ | This study |
| pKN18   | pQE-80L except Tsp lacO_lacO_ speA_speB_argA$^{ATG,Y19C}$ Tsp lacO_ lacO_speC | This study |
| pKT199  | pQE-80L except lacF$^R$ lacO_lacO_speA_speB_argA$^{ATG}$ Y19C lacI | This study |
| pKT200  | pQE-80L except lacF$^R$ lacO_lacO_speA_speB_argA$^{ATG}$ Y19C lacI | This study |
| pKT201  | pQE-80L except lacF$^R$ lacO_lacO_speA_speB_argA$^{ATG}$ Y19C lacI | This study |
| pQE-80L | CoIE1 replicon bla$^R$ lacF$^R$ Tsp lacO_lacO-(His)$^6$ | Qiagen |
| pSH1733 | pQE-80L except argA$^{ATG,Y19C}$ | This study |
| pSH1734 | pQE-80L except Tsp lacO_lacO_ speC$^+$ | This study |
| pSH1807 | pQE-80L except lacF$^R$ lacO_lacO_speA_speB_argA$^{ATG}$ Y19C lacI | This study |
| pSH1820 | pQE-80L except lacF$^R$ lacO_lacO_speA_speB_argA$^{ATG}$ Y19C lacI | This study |
| pSH1823 | pQE-80L except lacF$^R$ lacO_lacO_speA_speB_argA$^{ATG}$ Y19C lacI | This study |

Data analysis
All data were analyzed using SPSS Statistics 25 (IBM; Armonk, NY). The one-way analysis of variance (ANOVA) was used to determine significant differences.

Results
Effects of the initiation codon and Y19C substitution of ArgA
The wild-type strain, MG1655 excretes only 0.18 mM of putrescine in LB medium (data not shown). As arginine is a key substrate of putrescine synthesis through the ADC pathway, to increase putrescine production, we constructed plasmids with the ArgA desensitized to the feedback inhibition by arginine. In addition, the initiation codon of native ArgA is GTG, which is inefficient in translation initiation. Therefore, we generated a plasmid containing the argA gene with the effective ATG codon. Moreover, speA and speB genes were introduced under the lacF$^R$ promoter along with the argA gene in order to improve putrescine production. To compare the effects of both amino acid and initiation codon substitution, pSH1820 (argA$^{ATG,Y19C}$), pKT199 (argA$^{ATG,Y19C}$), pKT200 (argA$^{ATG,Y19C}$), and pKT201 (argA$^{ATG,Y19C}$) were constructed, and strain FS115 was transformed to obtain KT207, KT208, KT209, and KT210, respectively. The strains were grown in LB medium with ampicillin and incubated at 37 °C. The result is illustrated in Fig. 2. KT207 excreted the highest concentration of putrescine into the medium, whereas strain KT210 excreted the lowest amount of putrescine. To compare the effects of the initiation codon, the strains were separated into two groups: GTG codon (KT208 and KT210) and ATG codon (KT207 and KT209). ATG codon exhibited a higher concentration of putrescine than the original initiation codon, GTG. In addition, Y19C substitution in strains KT207 and KT208 resulted in notably higher putrescine
production than strains containing the native amino acid residue Y19 (KT209 and KT210).

**Effect of SpeA, SpeB, and SpeC on putrescine production**

There are ADC and ODC pathways for putrescine synthesis in *E. coli*. Whether the over-expression of ADC pathway only or of both ADC and ODC pathways is appropriate for the over-production of putrescine was not sure. To address this issue, we constructed a plasmid that has argA<sub>ATG Y19C</sub> together with only speC gene under T5 promoter (pFS29) and compare with another two plasmids which have argA<sub>ATG Y19C</sub> with only speAB genes (pKN11) and argA<sub>ATG Y19C</sub> with speAB and speC genes (pKN18). The plasmids were transformed into MG1655 as a host strain, resulting in FS123, KN24, and KN20, respectively. As depicted in Fig. 3A, the cell growth profiles of the above three strains were not different. However, the putrescine production of KN24, which has only speAB genes on a plasmid with argA<sub>ATG Y19C</sub> under T5 promoter, exhibited the highest putrescine content among these three strains (Fig. 3B). Therefore, the plasmid pKN11, which contains only speAB genes with argA<sub>ATG Y19C</sub>, was selected to use in further experiments.

**Effects of wild-type ArgA on ArgA<sub>ATG Y19C</sub>***

ArgA forms a homohexamer to be an active enzyme. When genomic argA is wild-type, it may form a mixed hexamer of ArgA<sub>wt</sub> and ArgA<sub>Y19C</sub>. ArgA<sub>wt</sub> may be dominant to ArgA<sub>Y19C</sub> in the desensitization of arginine. To compare the effects of wild-type ArgA, the strains with and without argA, FS115 (argA<sup>+</sup>) and KT32 (ΔargA), respectively, were constructed. The plasmid carrying speAB and argA (argA<sub>ATG Y19C</sub>) encoding desensitized ArgA<sub>ATG Y19C</sub> under the regulation of the lacI<sup>i</sup> promoter and lac operator was constructed (pSH1820) and used to transform the above-mentioned strains (FS115 and KT32), resulting in KT207 and KT39, respectively. To evaluate the effects of the coexistence of wild-type ArgA and desensitized ArgA<sub>ATG Y19C</sub>, KT207 and KT39 were used. As depicted in Fig. 4, the growth profile and putrescine production did not significantly differ between KT207 and KT39.
Effects of promoter strength on putrescine production

To promote the sequential reactions from arginine to putrescine, the promoter for speAB_argA_{ATG Y19C} was evaluated. The concentrations of IPTG for inducible lacI, lacI^p, lacI^q, and T5 promoters were compared. The lacI promoter of pSH1823 was replaced by lacI^p and lacI^q promoters, resulting in pSH1807 and pSH1820, respectively. The plasmid (pKN11) carrying the speAB_argA_{ATG Y19C} under the T5 promoter and two lac operators was constructed. Strain KT32 was transformed with each plasmid to obtain KT37 (lacI^p), KT38 (lacI^q promoter, open circle), KT39 (lacI^q1 promoter, closed square), and KT148 (T5 promoter, open diamond) grown in LB medium supplemented with 100 μg/mL of ampicillin. When the OD_{600} reached 0.4, 0.02 mM IPTG was added. Data shown are averages ± standard deviations, and culture experiments were performed in triplicate.

On the other hand, strains KT39 (lacI^q1p), and KT148 (T5p) produced 349% and 431% more putrescine than strain KT37 (lacI^p), respectively (Fig. 5B). Based on these results, KT148, carrying speAB and desensitized argA under the T5 promoter and two lac operators, was used for further study. To optimize the IPTG concentration, it was varied from 0 to 0.20 mM. Among these concentrations, 0.03 mM IPTG resulted in the highest putrescine production by KT148 in LB medium (data not shown).

Disruption of genomic puuPA increases putrescine production

To improve the accumulation of extracellular putrescine, the importer and degradation enzymes of putrescine
should be eliminated. The host strains KT32 (ΔpuuA ΔpatA), KT43 (ΔpuuAP ΔpatA), KT105 (puuP+ΔpatA), KT112 (ΔpuuAP patA+), and KT159 (puuP+ΔpatA) were constructed to assess the effects of patA, puuA, and puuP on putrescine production. The above strains were transformed by the plasmid pKN11 (T5p_speAB_αrgA ATG Y19C), leading to strains KT148, KT152, KT153, KT154, and KT162. Among all strains, puuP+ strains (KT153 and KT162) produced a lower amount of putrescine than the ΔpuuA (KT148) and ΔpuuAP (KT152 and KT154) strains (Fig. 6B). However, there were no significant differences in the concentration of extracellular putrescine between KT153 (ΔpatA) and KT162 (patA+) strains.

Effects of the medium on putrescine production
The amount of putrescine production was compared using LB medium and terrific broth to optimize the medium for putrescine production. Strain KT152 grew and produced putrescine more in terrific broth than in LB (Fig. 7). At 48 h of incubation, strain KT152 excreted 10.9 mM of extracellular putrescine in terrific broth, which was about twice as much as that in LB (Fig. 7B). Moreover, the IPTG concentration was optimized for cultivation in terrific broth. The concentration of IPTG was varied from 0 to 0.20 mM. The optimal concentration of IPTG for KT152 cultured in terrific broth was 0.02 mM (data not shown).

Effects of putrescine transporter PotFGHI and mutant YifEQ100TAG
To clarify whether the disruption of the PotFGHI transporter and the mutation on YifE improved putrescine production, strain SH2204 was transformed with yifE::FRT-kanR-FRT of SH2201 and the yifEQ100TAG was replaced using zie-296::TnJ0 as a co-transduction marker by P1 phage grown on SH2203. The potFGHI operon of the strain SH2204 (yifEQ100TAG) was disrupted by P1vir transduction to obtain strain SH2206 (yifEQ100TAG ΔpotFGHI). Strains KT43, SH2204, and SH2206 were transformed with pKN11 (T5p_speAB_αrgA ATG Y19C), and strains KT152, KT160, and KT161 were obtained. Putrescine production by these strains in a terrific broth with
the addition of 0.02 mM IPTG is compared in Fig. 8a. Samples were taken at 0, 2, 4, 6, 12, 24, 36, and 48 h of incubation. Among these sampling times, the highest concentration of putrescine was observed at 48 h (data not shown). There was no significant difference in putrescine production between strains KT160 and KT161. However, the amount of extracellular putrescine excreted from both KT160 (\(yifE^{Q100TAG}\) \(potFGHI^{+}\)) and KT161 (\(yifE^{Q100TAG}\) \(ΔpotFGHI\)) was 23% higher than that by KT152 (\(yifE^{+}\) \(potFGHI^{+}\)) (Fig. 8a). In addition, the IPTG concentration was optimized from 0 to 0.2 mM for the cultivation of KT160 in terrific broth. The optimal concentration of IPTG for KT160 cultured in terrific broth was 0.01 mM and the strain produced 19.8 mM of putrescine (Fig. 8b).

**Discussion**

Efficient initiation codon of \(argA^{Y19C}\) improved the putrescine production- Arginine is a key substrate of putrescine synthesis through the ADC pathway. The biosynthetic pathway of arginine initiates with glutamate to N-acetylglutamate by N-acetylglutamate synthase (ArgA), which is encoded by \(argA\). However, not only is the transcription of the \(argA\) gene repressed by ArgR, but the activity of ArgA is also inhibited by the final product arginine (Lu 2006; Sun et al. 2015; Xu and Zhang 2019a, b). To produce putrescine from glutamate, the repression of \(argA\) by ArgR and the feedback inhibition of ArgA by arginine are of concern. Previously, Rajagopal et al. (1998) constructed plasmids with a point mutation in the \(argA\) gene. Among all point mutations, the strain with the Y19C mutation, which is an amino acid substitution of tyrosine with cysteine at the 19th codon, was able to produce the highest amount of arginine. According to the review of Igarashi and Kashiwagi (2010), the addition of putrescine stimulates the translation of polyamine modulons. As ArgA is a polyamine modulon (Igarashi et al. 2015), its translation can be stimulated by the increase in putrescine. The effects of the substitution of the GTG codon with the ATG codon was evaluated, but the substitution of GTG with ATG was not so effective as expected (Fig. 2). This may be because the plasmid we constructed has only 8 base pairs between the SD sequence and the ATG initiation codon, and it lacks the characteristics of a polyamine modulon. However, desensitization of ArgA by amino acid substitution at 19 positions from tyrosine to cysteine from the plasmid increased putrescine production (Fig. 2). And the strain with \(argA^{ATG\ Y19C}\) had the highest amount of putrescine.

**The effects of wild-type ArgA on desensitized ArgA-** To confirm whether ArgA\(^{wt}\) or ArgA\(^{ATG\ Y19C}\) is dominant, we compared the putrescine production of KT39 (\(argA^{ATG\ Y19C}/ΔargA\)) and KT207 (\(argA^{ATG\ Y19C}/argA^{+}\)) strains. The growth and putrescine production of both KT39 and KT207 were not different. Thus, the presence of wild-type ArgA expressed from the genome does not affect the growth or putrescine production (Fig. 4).

**The strong promoter T5 increases putrescine production-** According to the report of Buch and Boyle (1985), a significant proportion of SpeA associates with the cell envelope. A recent paper reported that some of SpeA is in the periplasm and the other is in the cytoplasm (Meydan et al. 2019). Overexpression of membrane-associated or excreted proteins can affect cell health. Therefore, in this study, the promoter strength required for overexpression was of concern. The \(lacI\), \(lacI^\delta\), \(lacI^\phi\), and T5 promoters were compared. The strain carrying the \(lacI\)
promoter produced the lowest concentration of extracellular putrescine, whereas the strain carrying the T5 promoter produced the highest. Due to its affinity for RNA polymerase, the lacI promoter is the poorest (Muller-Hill et al. 1968). Therefore, the expression level is low. A point mutation at the −35 region of the lacI promoter led to tenfold higher expression and the lacFI promoter resulted in 170-fold higher expression (Calos 1978; Glasscock and Weickert 1998; Penunetcha et al. 2010). Moreover, there are two lac operators in this T5 promoter with a strong affinity to E. coli RNA polymerase (von Gabain and Bujard 1977, 1979; Shibui et al. 1988), but no study has directly compared their affinity for RNA polymerase. As depicted in Fig. 5B, the putrescine production of KT148 (T5p) was higher than that of KT39 (lacFI).

Effects of puuAP disruption on putrescine production- Two catabolic pathways of putrescine have been reported. PatA and PuuA are the first enzymes of the aminotransferase pathway and putrescine utilization pathway (the Puu pathway), respectively. PatA transfers one of the amino groups of putrescine to α-ketoglutarate (Shaibe et al. 1985; Schneider and Reitzer 2012). Another enzyme that catabolizes putrescine is PuuA, which γ-glutamylates the amino group of putrescine to generate γ-glutamylputrescine. The proteins involved in the Puu pathway are encoded in a gene cluster. The importer of this Puu pathway is PuuP, which is encoded in this gene cluster. After PuuP transports putrescine into the cell, the degradation step begins with PuuA (Kurihara et al. 2009b). There is no report to compare the reaction velocity or the expression level between the puuA and patA yet. So, it is difficult to specify which enzymes are mainly work. However, according to our previous study, the deletion of the puuA gene caused a severe effect on putrescine utilization and ΔpuuA strain could not grow using putrescine as a nitrogen source or a carbon source, while the deletion of the patA gene did not affect this issue (Kurihara et al. 2008). The result in this study is agreeable with the previous study that the presence of puuA showed the degradation of putrescine while patA is not. In addition, under the condition in which putrescine is present, PuuP is required as a putrescine transporter to use putrescine as an energy source (Kurihara et al. 2009b; Terui et al. 2014). As PuuP may also have its own promoter, we constructed KT148 (ΔpuuA) and KT152 (ΔpuuAP) to investigate the relationship between these puuAP genes. As shown in Fig. 6, puuAP may be an operon because ΔpuuAP had the same result as ΔpuuA. The deletion of puuA increased the extracellular accumulation of putrescine. In summary, PatA does not affect putrescine production, but the deletion of PuuA increases the amount of putrescine.

Terrific broth is a better medium for putrescine production- LB medium is widely used as a standard medium for a broad group of bacteria (Sezonov et al. 2007; Suzuki et al., 2019). However, the growth of E. coli is not high in the LB medium, whereas it is markedly high in terrific broth (Losen et al. 2004; Lessard 2013). Terrific broth contains 5.04 g/L of glycerol, while LB medium does not. E. coli can utilize glycerol as a carbon source for growth and putrescine production. Besides much higher concentration of yeast extracts, this is one of the reasons why the growth and putrescine production in terrific broth is better than those in LB medium (Fig. 7).

Mutation of YifE increases putrescine- During we attempted to find a putrescine exporter in E. coli K-12, we found that the extracellular putrescine concentration of strain JW0484 with ΔybbA::FRT-kanR-FRT was approximately double that of its parental strain, ME9062 (Additional file 1: Fig. S1). The ΔybbA::FRT-kanR-FRT was transduced to strain MG1655 to confirm the increase of putrescine production. However, the extracellular putrescine concentration of the MG1655 with ΔybbA::FRT-kanR-FRT was not different from that of MG1655. We predicted that the second mutation in strain JW0484 increased the extracellular putrescine concentration. Then, its genome sequence was performed to evaluate this prediction. The result of genome sequencing showed several mutations in strain JW0484, which were not reported in strain ME9062. Additional mutations were found in intA, fbaA, dgoR, yifE, and lexA in the genome of strain JW0484. Further experiments revealed that mutation of the yifE gene increases the extracellular putrescine concentration (Additional file 1: Fig. S2). As shown in Fig. 8a, the YifE mutant was also effective in our putrescine over-producing strain (KT160). RT-PCR revealed that the yifEQ100TTAG mutation increases the transcription of kdfR and gltB genes.

Pistocchi et al. (1993) reported that the PotFGHI transporter uptakes putrescine and accumulates it in the cell. Therefore, it is conceivable that PotFGHI reduces extracellular putrescine. We compared the strains with and without the PotFGHI transporter, but there was no difference in the concentration of putrescine between potFGHI+ and ΔpotFGHI strains. Thus, the PotFGHI transporter does not affect the amount of extracellular putrescine. Terui et al. (2014) reported that PotFGHI functions as a major transporter when putrescine is absent in the medium. However, the PotFGHI transporter can be inhibited by putrescine through a feedback loop. In our experimental condition, putrescine was synthesized and exported to the medium; therefore, PotFGHI may have been inhibited by putrescine. We achieved 19.8 mM of putrescine production from KT160 in terrific broth.
broth. When compare in terms of the product yield, Qian et al. (2009) reported a yield of putrescine at 30.3 g/M glucose, while our putrescine production is the 31.8 g/M glycerol. However, as putrescine is an ionic compound, its production altered the pH of the medium during cultivation. As we do not have a bioreactor device, we were unable to assess continuous feeding with pH adjustment.

Consent for publication
Not applicable.

Competing of interests
The authors declare that there is no conflict of interest.

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Additional file 1: Table S1. Primers used for strain construction. Table S2. Primers used for plasmid construction. Table S3. Primers used for Realtime PCR. Fig. S1. Comparison of extracellular putrescine concentration per OD600 among strains of the Keio collection. M9 glucose medium (60 mL in 100 mL Erlenmeyer flask with stirrer bar) was inoculated with the pre-culture at the initial OD600 of 0.06. The flasks were set in the 7-L rectangular anaerobic jar with 2 AnaeroPack-Anaero sachets (Mitsubishi Gas Chemical, Tokyo, Japan) to form an anaerobic environment and the jar was set on the stirrer in the microbiological incubator. The culture medium was stirred at 150 rpm and kept at 37°C. After 10 h of incubation, the OD600 of the culture was measured and 0.4 mL of the culture was centrifuged. Thirty μL of 100% (w/v) TCA was mixed with 0.3 mL of the supernatant, filtrated through the membrane filter, and then subjected to HPLC analysis as described in the main text. Asterisks indicate the genes with significant differences in transcription levels. Fig. S2. Q100TAG mutation in the yifE gene promotes putrescine production. Extracellular concentration of putrescine of AI32 (pQE-80L::MG1655, open diamond) and AI33 (pQE-80L::yifE100TAG/MG1655, closed square) cultured at 37°C in minimal M9 supplemented with 0.2% glucose. 100 μg/mL of ampicillin was added to maintain the plasmids. When the OD600 reached 0.5, the 0.5 mM IPTG was added. Fig. S3. The presence of hsdR had a slight, if any, effect on the increase in extracellular putrescine concentration by YifE100TAG. (A) Cell growth and (B) extracellular concentration of putrescine of AI32 (pQE-80L::MG1655, open circle), AI33 (pQE-80L::yifE100TAG/MG1655, closed triangle), and KT218 (pQE-80L::yifE100TAG/MG1655 but ΔhsdR::FRT-kan::FRT, closed square) cultured at 37°C in LB supplemented with 100 μg/mL of ampicillin. When the OD600 reached 0.4, 0.02 mM IPTG was added.

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Authors’ contributions
KT conducted all fermentation experiments in the main text and contributed to the interpretation, analysis and preparation of the draft. KI constructed a plasmid and performed the primary fermentation experiments. YS and AI identified the yifE mutant and found that the mutation can increase putrescine production. HS planned the study, acquired the funding, led the project, constructed some strains and plasmids, and edited the manuscript.

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Availability of data and materials
Some data are not shown in the text and supplement. However, they are available from the authors upon reasonable request. The strains and plasmids used in this work can be shared by exchanging MTA.

Declarations
Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.

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