Combined Effect of Improved Cell Yield and Increased Specific Productivity Enhances Recombinant Enzyme Production in Genome-Reduced *Bacillus subtilis* Strain MGB874

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**Summary**

Genome reduction strategies to create genetically improved cellular biosynthesis machineries for proteins and other products have been pursued by use of a wide range of bacteria. We reported previously that the novel *Bacillus subtilis* strain MGB874, which was derived from strain 168 and has a total genomic deletion of 874 kb (20.7%), exhibits enhanced production of recombinant enzymes. However, it was not clear how the genomic reduction resulted in elevated enzyme production. Here we report that deletion of the *rocDEF-rocR* region, which is involved in arginine degradation, contributes to enhanced enzyme production in strain MGB874. Deletion of the *rocDEF-rocR* region caused drastic changes in glutamate metabolism, leading to improved cell yields with maintenance of enzyme productivity. Notably, the specific enzyme productivity was higher in the reduced-genome strain, with or without the *rocDEF-rocR* region, than in wild-type strain 168. The high specific productivity in strain MGB874 is likely attributable to the higher expression levels of the target gene resulting from an increased promoter activity and plasmid copy number. Thus, the combined effects of the improved cell yield by deletion of the *rocDEF-rocR* region and the increased specific productivity by deletion of another gene(s) or the genomic reduction itself enhanced the production of recombinant enzymes in MGB874. Our findings represent a good starting point for the further improvement of *B. subtilis* reduced-genome strains as cell factories for the production of heterologous enzymes.

Due to recent advances in genetic engineering technology, a variety of useful substances, including enzymes, have been produced industrially by use of microorganisms. To further improve production efficiencies on an industrial scale, several approaches, such as mutational breeding, have been used to generate hyperproducing microbial cells. In addition, strategies for genome reduction (18), which represents a relatively new field in synthetic genomics, have been used with *Escherichia coli* (25, 44) and *Bacillus subtilis* (3, 39, 54) to investigate microbial genomic architecture and to improve characteristics relevant to protein production.

*B. subtilis*, a Gram-positive sporiferous bacillus, is an attractive organism for industrial use for a variety of reasons, including its high growth rate, protein secretion ability, and GRAS (generally regarded as safe) status (46, 49). *B. subtilis* is also one of the best-characterized model microorganisms, by biochemical, genetic, and molecular biological studies. Furthermore, the complete genomic sequence of *B. subtilis* strain 168 has been determined, facilitating genetic engineering of this industrially useful strain (4, 35).

In the 4.2-Mb genome of *B. subtilis* strain 168, only 271 of the 4,106 identified genes are indispensable for the growth of this organism in rich medium (33). In addition, *B. subtilis* has numerous genes that are activated only under specific conditions or in response to environmental stresses. Therefore, under controlled conditions, such as those typically encountered in industrial production systems, many genes are likely unnecessary and may be wasteful in terms of energy consumption. For the efficient production of heterologous enzymes, it may be beneficial to delete such unnecessary and wasteful genomic regions.

To date, several *B. subtilis* strains with reduced genomes have been engineered. For example, *B. subtilis* strain Δ6, with a 7.7% genome reduction (0.53 Mb), was reported by Westers et al. (54). However, phenotypic characterization of strain Δ6 cells revealed no unique properties relative to wild-type 168 cells. Recently, we constructed a multiple deletion mutant, MGB874, by the sequential deletion of 865 genes (874 kb; 20.7%) from the total genomic DNA of *B. subtilis* strain 168, including all prophage and prophage-like sequences, the *pps* operon, and 11 nonessential gene clusters (3, 39). Notably, compared to strain 168, strain MGB874 showed enhanced production of the exogenous extracellular alkaline cellulase Egl-237 (24) and the subtilisin-like alkaline protease M protease (31, 34) from plasmid-borne genes in modified 2xL-Mal medium, a model medium for industrial protein production. Although enzyme production in wild-type 168 cells was enhanced by deletion of the *rocDEF-rocR* region, which is involved in arginine degradation, contributes to enhanced enzyme production in strain MGB874. Deletion of the *rocDEF-rocR* region caused drastic changes in glutamate metabolism, leading to improved cell yields with maintenance of enzyme productivity. Notably, the specific enzyme productivity was higher in the reduced-genome strain, with or without the *rocDEF-rocR* region, than in wild-type strain 168. The high specific productivity in strain MGB874 is likely attributable to the higher expression levels of the target gene resulting from an increased promoter activity and plasmid copy number. Thus, the combined effects of the improved cell yield by deletion of the *rocDEF-rocR* region and the increased specific productivity by deletion of another gene(s) or the genomic reduction itself enhanced the production of recombinant enzymes in MGB874. Our findings represent a good starting point for the further improvement of *B. subtilis* reduced-genome strains as cell factories for the production of heterologous enzymes.

† Supplemental material for this article may be found at http://aem.asm.org.

‡ K.M. and Y.K. contributed equally to this work.

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strain MGB874 and investigated the mechanism of enhanced which gene deletions elevated the enzyme production levels in order to provide insights into approaches for further improve-
gous enzymes.

Moreover, it is unclear how these continued to increase in strain MGB874 throughout the culture arrested after the transition state, the production levels con-
tinued to increase in strain MGB874 throughout the culture period (39). In addition, transcriptome analyses of strain MGB874 revealed earlier development of genetic competence, delayed entry into sporulation, and prolonged maintenance of metabolic activity (39). However, it is unclear how these changes contribute to the increased production of heterolo-
gous enzymes.

The aim of this study was to reveal the mechanisms under-
lying the enhanced enzyme production in strain MGB874 in order to provide insights into approaches for further improvements of this strain. Specifically, we attempted to determine which gene deletions elevated the enzyme production levels in strain MGB874 and investigated the mechanism of enhanced enzyme production by focusing on two factors: specific productivity and cell yield.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth media.** The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* HB101 (Takara Bio, Inc.) was used as the host for plasmid preparation and was routinely cultured in Luria-Bertani (LB) medium (1% [wt/vol] Bacto tryptone [Difco], 0.5% [wt/vol] Bacto yeast extract [Difco], and 1% [wt/vol] NaCl). For preparation of *B. subtilis* competent cells, Spizizen minimal medium (2) was used as the basal medium. LB medium containing 1.5% (wt/vol) agar and supplemented with 100 μg ml⁻¹ ampicillin, 10 μg ml⁻¹ chloramphenicol (CM), 20 μg ml⁻¹ neomycin (Nm), 100 μg ml⁻¹ spectinomycin (Sp), or 0.3 μg ml⁻¹ erythromycin (Em) was used as the selective medium. The protoplast transformation method (14) was used for the introduction of plasmids into *B. subtilis*, and the transformants were selected on

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**TABLE 1. Bacterial strains and plasmids used or constructed in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
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<td></td>
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<tr>
<td>Bacillus subtilis strains</td>
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<td>168</td>
<td>trpC2</td>
<td>35</td>
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<tr>
<td>MGB625</td>
<td>Δprophage 1-6 ΔPBSX ΔSPB Δpsk Δskin Δppx Δ(ydeK-ydhU) Δ(yisB-yilD) Δ(yuaA-yurT) Δ(gcpE-yodU) Δ(yphP-yopQ) Δ(yek-yveX)</td>
<td>39</td>
</tr>
<tr>
<td>MGB723</td>
<td>MGB625 Δ(pd-p-rocR)</td>
<td>39</td>
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<tr>
<td>MGB874</td>
<td>MGB723 Δ(roc-rocR) Δ(yokS-ryaK) Δ(sboA-yswhH) Δ(yphP-ysylH) Δ(yncM-ysydN)</td>
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</tr>
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<td>MGB625 Δr1</td>
<td>MGB625 Δ(pd-p-rocD):cat</td>
<td>This study</td>
</tr>
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<td>MGB625 Δr2</td>
<td>MGB625 Δ(yaeC-yaanA):cat</td>
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</tr>
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<td>MGB625 Δr3</td>
<td>MGB625 Δ(yacD-bfp):cat</td>
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<td>MGB625 Δ(ydd-rocR):cat</td>
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<td>MGB874 with cat-roc-DEFrocr inserted at the Δ(pd-p-rocR) locus</td>
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<tr>
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<td>pHYS237</td>
<td>Shuttle vector for <em>E. coli</em> and <em>B. subtilis</em></td>
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<td>pH2P37-K16</td>
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<td>pX</td>
<td>Integration vector containing the Px promoter used for integration into the amyE locus by double crossover using cat</td>
<td>34</td>
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* Antibiotic resistance genes are shown as follows: cat, chloramphenicol; spec, spectinomycin; neo, neomycin; erm, erythromycin; amp, ampicillin; tet, tetracycline.
FIG. 1. Determination of the deletion contributing to enhanced enzyme production in reduced-genome strains of B. subtilis. (A) The region (pdp to rocR) deleted from MGB625 to construct MGB723. The newly deleted regions (r1, r2, r3, and r4) of MGB625 are indicated with arrows. (B) Clusters of genes contained in the r4 region. Arrows indicate the regions deleted from MGB625. (C and D) Cellulase activities in the growth media of B. subtilis derivative strains harboring pHYS237 for Egl-237 production. (E) Protease activities in the growth media of B. subtilis derivative strains harboring pHP237-K16 for M protease production. Cells were precultured in LB medium containing 15 μg/ml Tet at 30°C for 15 h and were inoculated into 2×L-Mal medium. Cells further cultured at 30°C for 75 h were separated from the growth medium by centrifugation, and the supernatants were used for determinations of cellulase and protease activities. The results presented are the means for three individual experiments. Error bars represent standard deviations (n = 3).

Construction of B. subtilis mutant strains. The DNA manipulation techniques used for the construction of mutants were described previously (36). MGB625 Δr1 was constructed from MGB625 as follows. A 0.9-kb Cm resistance gene (cat) fragment was PCR amplified from plasmid pC194 (26) by using primers CmFW and CmRV. Approximately 1.0-kb upstream (UPr1) and downstream (DNr1) flanking sequences of the r1 region, which included the region from pdp to yxdD, were amplified using primers pdpFW plus pdp/Cmr and pdp/CmF plus pdp/RV, respectively. The two amplified fragments (UPr1 and DNr1) contained overlapping sequences with 5′ and 3′ ends, respectively. The two amplified fragments were mixed and fused together by splicing using overlap extension PCR (SOE-PCR) (27) with primers S237p-F.BamHI and S237pMpm-R (see Table S1). The 1.3-kb and 0.6-kb fragments were then ligated together in the order UPr1-cat-DNr1 by SOE-PCR using primers r1FW and r1RV, which were designed using internal sequences of the fragments to minimize the minor products of PCR ligation. The resulting 2.9-kb PCR product was then introduced into B. subtilis MGB625 cells by competent cell transformation (2), and Cm-resistant transformants were selected from LB agar plates containing Cm. Deletion of the r1 region was confirmed by PCR using primers r1FW and r1RV. A similar approach was used for the construction of mutant strains MGB625 Δr2, MGB625 Δr3, MGB625 Δr4, MGB625 ΔyydD-yxeC, MGB625 Δpdp-phrG, MGB625 ΔrocF-rocR, MGB625 ΔrocF-rocD, MGB625 ΔrocR, and MGB625 Δpdp-phrG, using the primers listed in Table S1 in the supplemental material. The r2, r3, and r4 regions represent the regions carrying yxeC to yxeA (nucleotides 4,048,987 to 4,066,589 in NCBI GenBank sequence NC_000964.3 [4]), were PCR amplified from genomic DNA of B. subtilis strain MGB625 by using primers r2FW plus r2/CmR and r2/CmF plus r2RV, respectively. The two amplified fragments (UPr1 and DNr1) contained overlapping sequences with cat at the 3′ and 5′ ends, respectively. The three fragments (cat, UPr1, and DNr1) were then ligated together in the order UPr1-cat-DNr1 by SOE-PCR using primers r1FW and r1RV, which were designed using internal sequences of the fragments to minimize the minor products of PCR ligation. The resulting 2.9-kb PCR product was then introduced into B. subtilis MGB625 cells by competent cell transformation (2), and Cm-resistant transformants were selected from LB agar plates containing Cm. Deletion of the r1 region was confirmed by PCR using primers r1FW and r1RV. A similar approach was used for the construction of mutant strains MGB625 Δr1, MGB625 Δr2, MGB625 Δr3, MGB625 Δr4, MGB625 ΔyydD-yxeC, MGB625 Δpdp-phrG, MGB625 ΔrocF-rocR, MGB625 ΔrocF-rocD, MGB625 ΔrocR, and MGB625 Δpdp-phrG, using the primers listed in Table S1 in the supplemental material. The r2, r3, and r4 regions represent the regions carrying yxeC to yxeA (nucleotides 4,048,987 to 4,066,582 in NCBI GenBank sequence NC_000964.3), respectively (1A).

Strain 874DEFR was constructed by transformation of strain MGB874 with chromosomal DNA from strain MGB625 Δpdp-phrG. As a result of the trans-
formation, the recDEF-recR region and cat were reintroduced into the ΔgudB-recR region in strain MG874. PCR was used to confirm that only the recDEF-recR region and cat were introduced into the resulting strain, 874DEFr. In addition, strain 874DEFr(spc) was constructed by replacement of cat with the Sp resistance gene (spc) in strain 874DEFr. The Sp resistance gene was amplified from plasmid pDG1727 (23) by using primers Spf and Spr. Strain 168 ΔgudB was constructed from strain 168 by using a slightly modified method from that used for the construction of strain MGB26 ΔargF. Briefly, a 0.8-kb fragment of the Nm resistance gene (neo) was first amplified from plasmid pUB110 (38) by using primers NmF and Nmr. Approximately 1.0-kb upstream (UPgudB) and downstream (DNgudB) flanking sequences of the coding region of gudB were PCR amplified from strain 168 genomic DNA by using primers gudBF plus gudB/Nmr and gudB/NmF plus gudBRV, respectively. The three amplified fragments (neo, UPgudB, and DNgudB) were ligated by SOE-PCR using primers gudBF2 and gudBRV, and template DNA was then removed by digestion with DpnI (Takara Bio, Inc.). The resulting 3.0-kb PCR product was introduced into B. subtilis strains 168, MG874, and 874DEFr, and Nm-resistant transformants were selected on LB agar plates containing Nm and designated strains 168 ΔgudB, MG874 ΔgudB, and 874DEFr ΔgudB, respectively. Successful deletion of the gudB region was confirmed by PCR using primers gudBF and gudBRV. A similar approach was used for the construction of mutant strains MG874 ΔrocG, MG874 ΔargF-argR, MG874 ΔargC-argR, MG874 ΔΔargF, MG874 ΔΔargC, MG874 ΔΔargF-argR, MG874 ΔΔrocG, MG874 ΔΔrocG-rocR, MG874 ΔΔrocG-rocR-argF-argR, MG874 ΔΔrocG-rocR-argF-argR-argC-argR, MG874 ΔΔrocG-rocR-argF-argR-argC-argR-cat-amyE and MG874 ΔΔrocG-rocR-argF-argR-argC-argR-cat-amyE-fur. The three fragments (gudB, argC-argR, and rocG-rocR), using the primers listed in Table S1 in the supplemental material. The Em resistance gene (erm) and promoter- and terminatorless Nm resistance gene were amplified from plasmids pMUTIN4 (53) and pUB110, respectively, using the primer sets Spf plus Spr, Emf plus Emr, and NmF plus Nmr2, respectively. The three ΔΔargF-argR strains, the coding region of abch was replaced by a promoter- and terminatorless Nm resistance gene to avoid influencing the expression of genes downstream of gudB.

The insertion of a 3.1-kb egf-237 fragment consisting of the promoter, SD sequence, and coding region for the signal sequence, mature protein, and terminator of egf-237 into the amyE locus of the B. subtilis chromosome was performed as follows. The 3.1-kb egf-237 fragment was amplified from plasmid pHYS237 by using primers amyE-e237r and amyE-egf237r. The front sequence of amyE (amyE-front) and its back sequence fused to cat (cat-amyE-back) were PCR amplified from plasmid PX (32) by using primer pairs amyE-up-F plus amyE-up-R and amyE-down-F plus amyE-down-R, respectively. The three fragments (egf-237, amyE-front, and cat-amyE-back) were ligated by SOE-PCR using primers amyE-up-F and amyE-down-R, and template DNA was then removed by DpnI digestion. The obtained PCR products were introduced into B. subtilis strains 168, MG874, and 874DEFr(spc), and Cm-resistant transformants were selected on LB agar plates containing Cm and designated strains 168egf237, MG874egf237, and 874DEFre237, respectively. Successful introduction of egf-237 was confirmed by PCR using primers amyE-up-F and amyE-down-R.

Culture methods for assessment of heterologous enzyme production. B. subtilis mutants were transformed with pHYS237 or pHPS237-K16 by the protoplast transformation method (14). To assess the production levels of heterologous enzymes, two culture methods were used. In the first method, which was used only for the evaluation of enzyme production levels, cells were cultured in 10 ml LB medium supplemented with 15 μg ml⁻¹ Tct with shaking at 120 rpm at 30°C for 15 h, and 600 μl of the pre-culture broth was inoculated into 30 ml 2×LB-Mal medium in a 500-ml Sakaguchi flask. After further cultivation with shaking at 120 rpm at 30°C for 72 h, cells were removed by centrifugation at 9,000 × g, and cellulase and protease activities in the culture supernatant were measured. In the second culture method, which was used for time course experiments, cells of strains 168, 874DEFr, and 874DEFr harboring pHYS237 that were stored in 10% glycerol at −80°C were inoculated into LB agar medium supplemented with 15 μg ml⁻¹ Tet. After incubation at 37°C for 12 h, cells were harvested from the plate surface and then inoculated into 30 ml 2×LB-Mal pre-culture medium at an optical density at 600 nm (OD₆₀₀) of 0.1 to 0.2. The cells were further cultured with shaking at 120 rpm at 30°C until reaching an OD₆₀₀ of 0.3 to 0.5, and then treated to an OD₆₀₀ of 0.30 with 0 ml 2×LB-Mal medium in a 500-ml Sakaguchi flask with further fermentation with shaking at 120 rpm at 30°C. At several time points, cells were removed by centrifugation at 9,000 × g and culture supernatants were collected and stored at −30°C until used for assays. For RNA extraction, cells separated by centrifugation were washed with 10 mM Tris-HCl (pH 7.5), frozen with liquid nitrogen, and then stored at −80°C.

Viable cell counts. The detection of viable bacteria was performed by cultivation and enumeration of CFU. A dilution series of bacterial cell suspensions was plated on LB agar plates. After overnight incubation at 37°C, colonies formed on the agar plates were enumerated.

Assay of enzyme activity. For the determination of cellular cellulase activity, 50-μl aliquots of 0.4 μM p-nitrophenyl-β-D-cellobioside (Seikagaku Kogyo) were mixed with 50 μl sample solution appropriately diluted with 130 mM phosphate buffer (pH 7.4) (Wako Pure Chemical Industries). The amount of p-nitrophenol released during a reaction at 30°C was then determined quantitatively based on the change in absorbance at 420 nm (A₄₂₀). The amount of enzyme required for the release of 1 μmol p-nitrophenol per min was defined as 1 U. Specific productivity (U g⁻¹ h⁻¹) was calculated by dividing the cellulase production rate (U liter⁻¹ h⁻¹) by the cell dry weight (CDW) (g liter⁻¹).

Measurement of extracellular amino acid concentrations. The collected culture supernatants were first diluted 21-fold and suspended in 2% (wt/vol) tri-chloroacetic acid. After the precipitate was removed by filtration through a 0.2-μm cellulose acetate filter (DISMIC-13CP, Advantec), the filtrates were subjected to determination of amino acid concentrations by use of a model L-8900 amino acid analyzer (Hitachi).

Measurement of intracellular amino acid concentrations. Metabolites were extracted from cells according to the method described by Saga et al. (51) and Bolten et al. (12). Briefly, culture medium corresponding to 10 OD₆₀₀ units (e.g., 10 ml of a culture with an OD₆₀₀ of 1.0) was passed through a 0.45-μm Millipore filter (Millipore). Cells retained on the filter were washed twice with 1% (wt/vol) NaCl and then immersed in 2 ml methanol containing 150 μM m-aminobutyrate as an internal standard. After incubation for 30 min on ice, 1 ml chloroform and 380 μl Milli-Q water were added to the cell suspension, and 1 ml of the resulting methanol-water layer was filtered centrifugally through a Microcon YM-3 filter (Millipore). After drying the filter under reduced pressure at room temperature, amino acids were quantified using an HP6890 series gas chromatography (GC) system (Hewlett Packard) for GC detection-flame ionization (EZ:faast, Phenomenex) or a model L-8900 amino acid analyzer (Hitachi). The intracellular amino acid levels were calculated by determining the amount of amino acids per mg of CDW.

DNA sequencing. DNA sequencing was performed using the dideoxy chain termination method with a BigDye Terminator v3.1 cycle sequencing kit and an ABI 3100 sequencer (Applied Biosystems). If necessary, the target regions were amplified using specific primers and the PCR products were used as templates for sequencing. Primers for sequencing were designed according to the manufacturer’s instructions.

qRT-PCR. Total RNA was extracted from B. subtilis cells as described previously (28) and then reverse transcribed to cDNA by use of an AffinityScript QPCR cDNA synthesis kit (Stratagene). As negative controls, all RNA samples were subjected to identical reaction conditions without reverse transcriptase. Quantitative real-time PCR (qRT-PCR) amplification, detection, and analysis were performed with an Mx3000 real-time PCR system (Stratagene) and Brilliant II Fast SYBR green QPCR master mix (Stratagene). The primer sequences used in the real-time PCR were designed using Primer3 (version 0.4.0) (45) and are listed in Table S2 in the supplemental material. The source code for Primer3 is available at http://frodo.wi.mit.edu/Primer3/.

Real-time PCR was performed in 25 μl reaction mixtures consisting of 1× SYBR green master mix, 0.4 μM (each) forward and reverse primers, 3.2 μM reference dye, and 10 μl template. The PCR conditions were 95°C for 2 min followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. All of the amplified products were confirmed by dissociation curve analysis. To estimate the quantity of initial template in the sample, serial real-time PCRs were performed by amplifying inserted target DNA in pUC118 (Takara Bio, Inc.) by use of specific primers. For each gene, a standard curve was generated by using the log of the quantity of initial template DNA plotted against the threshold cycle (Cₚ) values for the standard wells. The generated standard curves were used to convert the Cₚ values for each amplified gene in the cDNA preparations to copy numbers of cDNA molecules. The estimated copy number was normalized to the value for
FIG. 2. Growth characteristics of strains 168, MGB874, and 874DEFR producing Egl-237 cellulase. Strains 168 (white circles), MGB874 (black triangles), and 874DEFR (gray squares) were transformed with pHYS237 and then cultured for time course experiments in 2×L-Mal medium at 30°C with shaking. (A) Growth profiles of the three strains. (B) Cellulase activities in the growth medium. (C) Specific productivities (U g⁻¹ h⁻¹) for strains 168, MGB874, and 874DEFR, calculated after 40 h of culture. Specific productivity (U g⁻¹ h⁻¹) was calculated by dividing the cellulase production rate (U liter⁻¹ h⁻¹) by the CDW (g liter⁻¹). The results presented are the means for three individual experiments. Error bars represent standard deviations (n = 3).

**RESULTS**

**Determination of gene deletions contributing to enhanced enzyme production.** Previously, we constructed a multiple deletion mutant, strain MGB874, by stepwise introduction of 23 large-scale deletions of genomic regions, and found that exogenous enzyme production increased in proportion to the genomic deletion size (39). In addition, we detected a significant increase in the enzyme production level of strain MGB723, which was generated from strain MGB625 by deletion of the region from pdp to rocR (pdp-rocR region; nucleotides 4,049,059 to 4,147,133 in NCBI GenBank sequence NC_000964.3) (Fig. 1A). Here we attempted to determine which genes deleted in this region were involved in the enhanced enzyme production.

First, the pdp-rocR region was divided into four regions, r1, r2, r3, and r4 (Fig. 1A), and each region was deleted individually from strain MGB625. The resulting mutant strains were transformed with pHYS237 to evaluate production of the cellulase Egl-237 (24). Since cellulase activity was not detected in the culture broth (Fig. 1C), we next evaluated Egl-237 production in strain 874DEFR, which was constructed by reintroducing rocDEF and rocR into MGB874, and found that cellulase activity in the growth medium was considerably lower than that detected for MGB874 and was nearly equivalent to that of wild-type strain 168 (Fig. 1D). Conversely, Egl-237 production by strain 168 ∆rocDEF-rocR, in which the rocDEF-rocR region was deleted, was approximately 1.2-fold higher than that by parental strain 168, although Egl-237 production by strain MGB874 was approximately 1.6-fold higher than that by strain 874DEFR (Fig. 1D). These data suggest that one of the 22 other regions missing from the 874DEFR genome or the extensive genome reduction itself enhanced the positive effect of the deletion of rocDEF-rocR on Egl-237 production in reduced-genome strain MGB874.

To verify whether deletion of the rocDEF-rocR region influenced the production of other enzymes, the levels of the alkaline protease M protease were evaluated. As indicated in Fig. 1E, the production of M protease was also improved by deletion of the rocDEF-rocR region.

**Changes in cell yield and specific enzyme productivity caused by rocDEF-rocR deletion.** To obtain insight into the enhancement of exogenous enzyme production by strain MGB874, we conducted time course analyses of cell yield and Egl-237 production. Cell yield was determined by measurement of the OD₆₀₀ of the culture medium, which was correlated to CDW. As we reported previously (39), the level of Egl-237 production in strain MGB874 was higher than that in strain 168, whereas the rates and yields of cell growth were
comparable (Fig. 2A and B). Thus, specific productivity (U g\(^{-1}\) h\(^{-1}\)), which was calculated by dividing the cellulase production rate (U liter\(^{-1}\) h\(^{-1}\)) by CDW (g liter\(^{-1}\)), was higher for strain MGB874 than for strain 168 (Fig. 2C). In contrast, the cell yield at stationary phase and the level of Egl-237 production in strain 874DEFR were lower than those in parental strain MGB74 (Fig. 2A and B). However, the specific productivity (U g\(^{-1}\) h\(^{-1}\)) of strain 874DEFR was nearly equal to that of strain MGB874 and was higher than that of strain 168 (Fig. 2C). The viable cell counts at stationary phase (40 h) were proportional to the cell yields for strains 168, MGB874, and 874DEFR (2.48 \(\times\) 10\(^{10}\), 2.23 \(\times\) 10\(^{10}\), and 2.32 \(\times\) 10\(^{10}\) CFU ml\(^{-1}\) OD\(_{600}\) unit\(^{-1}\), respectively). Collectively, the specific productivities of the reduced-genome strains (MGB874 and 874DEFR) were higher than that for wild-type strain 168, although strain 874DEFR had a lower cell yield than strains 168 and MGB874. These results indicated that the rocDEF-rocR deletion increased the cell yield of strain MGB874, allowing the cell yield of strain 168 to be reached, and that the deletion of another gene(s) or the genomic reduction itself was responsible for the higher specific productivity in strain MGB874. Therefore, the rocDEF-rocR deletion compensated for the decrease of cell yield that resulted from a prior deletion(s). Supporting these findings, the cell yield of strain MGB625 was lower than that of strain 168, and deletion of the rocDEF-rocR region in strain MGB625 increased the cell yield, with maintenance of a high specific enzyme productivity that was almost equal to that of strain MGB874 (see Fig. S1 in the supplemental material). Interestingly, deletion of the rocDEF-rocR region in strain 168 also increased the cell yield while maintaining the specific enzyme productivity (see Fig. S1 in the supplemental material).

Molecular basis of enhanced Egl-237 production. To determine whether the high specific productivities of Egl-237 in strains MGB874 and 874DEFR resulted from elevated mRNA levels, we determined the transcriptional levels of the Egl-237 gene (egl-237) and found that the levels of egl-237 transcripts were higher for strains MGB874 and 874DEFR than for strain 168 during stationary phase (Fig. 3A). Additionally, the egl-237 transcript level from plasmid pHYS237 in strain MGB874 was slightly higher than that in strain 874DEFR (Fig. 3A).

We also examined the pHYS237 plasmid copy number and promoter activity of egl-237. To estimate the promoter activity, we inserted a single copy of egl-237 into the chromosomal amyE locus in strains 168, MGB874, and 874DEFR and designated the resulting mutant strains 168egl-237, MGB874egl-237, and 874DEFRegl-237, respectively. As indicated in Fig. 3B and C, the plasmid copy numbers and transcript levels, respectively, from a single copy of egl-237 in the reduced-genome strains were significantly higher than those in the wild-type strain during stationary phase. In addition, pHYS237 copy number and egl-237 promoter activity in strain MGB874 were slightly higher than those in strain 874DEFR (Fig. 3B and C).

The plasmid copy numbers in strains 168, MGB874, and 874DEFR harboring multicopy plasmid pHYS237 were determined to be 5.0 \(\times\) 10\(^{2}\), 1.6 \(\times\) 10\(^{2}\), and 1.3 \(\times\) 10\(^{2}\) copies per cell, respectively, and the transcript levels from a single copy of egl-237 in strains 168egl-237, MGB874egl-237, and 874DEFRegl-237 were found to be 2.8\(\times\), 1.5 \(\times\) 10\(^{-1}\), and 8.4-fold higher, respectively, than that of gyrA (Fig. 3B and C). Thus, the estimated relative transcript levels of egl-237, which were calculated by multiplying the plasmid copy number by the transcript level from a single copy of egl-237, were 1.4 \(\times\) 10\(^{-2}\), 2.4 \(\times\) 10\(^{-3}\), and 1.1 \(\times\) 10\(^{-3}\) for strains 168, MGB874, and 874DEFR harboring pHYS237, respectively. However, the actual values were only 1.1 \(\times\) 10\(^{-2}\), 3.5 \(\times\) 10\(^{-3}\), and 2.6 \(\times\) 10\(^{-3}\), respectively (Fig. 3A, 24 h), which were lower than expected, suggesting that the transcript levels of egl-237 in the reduced-genome strains might be saturated. Evaluation of the growth and enzyme characteristics of strains 168egl-237, MGB874egl-237, and 874DEFRegl-237 showed that higher specific enzyme productivities were also observed for a single copy of the egl-237 gene in the reduced-genome strains, although the absolute cellulase production levels were decreased to approximately 1/10 those in strains harboring pHYS237 (see Fig. S2 in the supplemental material).

Metabolic changes caused by rocDEF-rocR deletion in strain MGB874. RocR is a positive regulator of genes related to the arginine degradation pathway involving RocG (a major catalytic glutamate dehydrogenase) (1, 7, 8, 13, 21, 22), which also serves as a regulatory protein that inhibits GltC (15), a transcriptional regulator specific to the gltAB operon, encoding the
large and small subunits of glutamate synthase (GOGAT) (11, 42) (Fig. 4).

To investigate the influence of deletion of the rocDEF-rocR region on carbon and nitrogen metabolism, we measured extracellular and intracellular amino acid levels (Fig. 5). Among the amino acids analyzed in the culture broth, a significant difference between the strains was detected only for arginine (Fig. 5A). In the culture broths of strains 168 and 874DEFR, both arginine and glutamate were depleted before cells entered the stationary phase (Fig. 5A). In contrast, in the culture medium of strain MGB874, the arginine level decreased gradually throughout the culture period due to the inactivation of the arginine degradation pathway. Glutamine was below the limit of detection in the culture broths of strains 168, MGB874, and 874DEFR.

Additionally, to investigate the influence of deletion of the rocDEF-rocR region on intracellular amino acid levels, cells were harvested from each culture at 16 h (Fig. 5A, arrows a) and 40 h (Fig. 5A, arrows b) and were subjected to amino acid analyses (Fig. 5B). At the two examined time points, the intracellular glutamate level in strain MGB874 cells was significantly larger than those in strain 168 and 874DEFR cells ($P < 0.05$). However, the intracellular glutamine level in strain MGB874 cells was marginally higher than that in strain 168 cells at 16 h ($P < 0.1$) and was significantly lower than those in the other two strains at 40 h ($P < 0.05$). The intracellular arginine levels were below the limit of detection.

**Expression levels of genes related to arginine and glutamate metabolism.** To understand the influence of deletion of the rocDEF-rocR region on metabolism in greater detail, changes in the expression levels of genes related to arginine and glutamate metabolism were monitored (Fig. 6). RNA samples prepared from the cultures of strains 168, MGB874, and 874DEFR at different stages of growth were subjected to qRT-PCR analysis using the specific primer sets shown in Table S2 in the supplemental material. The metabolic changes in MGB874 cells are illustrated in Fig. 7, with reference to these results and the data from tiling array analyses reported previously (39).

(i) Arginine metabolism. In strain MGB874 cells, deletion of the rocDEF-rocR region causes impairment of the arginine degradation pathway, and the arginine remaining in the culture medium will likely lead to repression of the arginine synthetic pathway by activation of the transcriptional factor AhrC (5). Indeed, we found that the expression levels of rocA and rocD, located in the rocABC and rocDEF operons, respectively,
which are related to the arginine degradation pathway, and argC of the argCJBD-carAB-argF operon, which is related to the arginine synthetic pathway, were markedly low in strain MGB874 cells throughout the culture period (Fig. 6). In contrast, in strain 168 and 874DEFR cells, rocA and rocD were expressed transiently during the arginine-consuming phase, and argC was expressed after depletion of arginine in the culture broth (Fig. 5A and 6). Since arginine synthetic enzymes convert glutamate to arginine-related compounds (Fig. 7), their repression in strain MGB874 cells contributes to maintenance of intracellular glutamate levels (Fig. 5B).

(ii) Aconitase. The expression of citB was reported to be repressed by arginine catabolism (10), during which 2-oxoglutarate generated from arginine competitively represses the reaction of citrate synthase (CitZ), leading to the repression of citB by CcpC in the absence of the effector citrate (29, 52) (Fig. 7). Consistent with this previous observation (10), the transcriptional level of citB in MGB874 cells was found to be 2-fold higher than that in 874DEFR cells at 24 h (Fig. 6). Unexpectedly, however, after 24 h of culture, the expression level of citB in 874DEFR was higher than that in 168 cells (Fig. 6), despite complete restoration of the arginine degradation pathway in

![Figure 6. Time course of transcription during growth of B. subtilis.](http://aem.asm.org/)

![Figure 7. Illustration of proposed intersection between nitrogen and carbon metabolism in MGB874 during stationary phase.](http://aem.asm.org/)
strain 874DEFR. Although the mechanism is unclear, the deletion of the rocDEF-rocR region appears to have contributed partly to the high level of citB expression in MGB874. It is possible that the activation of the citB aconitase gene might contribute not only to the generation of reducing power through the tricarboxylic acid (TCA) cycle but also to improvement of metabolic flux from carbon sources to glutamate synthesis (Fig. 7).

(iii) Glutamate dehydrogenase. The major glutamate dehydrogenase RocG was strongly repressed in strain MGB874 cells, as expected by deletion of the rocR gene (Fig. 6). In contrast, a second cryptic glutamate dehydrogenase gene, gudB, was expressed constitutively in strain 168, MGB874, and 874DEFR cells (Fig. 6). Previously, Belitsky and Sonenshein (7) reported that the GudB protein in laboratory Bacillus subtilis strains contains an insertion of 3 amino acids (9 bp) with respect to the common ancestral GluDH sequence and that the gudB genes present in the strains contain an essential gene for cell growth in Bacillus subtilis under the cultivation conditions used in this study.

(ii) Aconitase. The deletion of citB caused severe growth inhibition in the reduced-genome strains MGB874 and 874DEFR; thus, the direct contribution of citB expression to Egl-237 production could not be investigated (Fig. 8B). Growth inhibition following citB inactivation also occurred in wild-type strain 168, suggesting that citB is an essential gene for cell growth in Bacillus subtilis under the cultivation conditions used in this study.

(iii) Glutamate dehydrogenase. The deletion of rocG resulted in increases in cell yield in both MGB874 and 874DEFR (Fig. 8C). However, specific productivities were decreased significantly in these rocG mutants. We found that this phenomenon was due to an impairment of Egl-237 secretion caused by a drastic decrease of external pH in the growth medium of the rocG mutants and that the high specific productivities in these strains could be maintained by controlling the external pH (results to be published elsewhere).

Belitsky et al. (6) reported that even in the absence of the activator RocR, rocG is expressed due to readthrough transcription from the upstream yweA promoter. Therefore, rocG is likely expressed at low levels in strain MGB874. The low-level expression of rocG is expected to play a crucial role in the control of cell yield, as the deletion of rocG increased the cell yield of strain MGB874 (Fig. 8C). In addition, the deletion of rocG in strain 874DEFR significantly increased the cell yield to a level similar to that of strain MGB874 (rocG). Based on these results, the decreased expression of rocG by deletion of the rocR gene appears to be the main underlying factor for improving the cell yield of strain MGB874.

(iv) GS-GOGAT pathway. In MGB874 cells, the first gene of the GOGAT operon, gltAB, was expressed abundantly (Fig. 6), likely due to the repression of rocG expression (Fig. 4 and 7). The activation of gltA may have contributed significantly to the increase in intracellular glutamate levels observed during stationary phase (Fig. 5B). The transcriptional levels of the glutamine synthetase (GS) gene glnA were also higher for strain MGB874 than for strains 168 and 874DEFR at 24 and 40 h (Fig. 6). Upon activation of the glutamate synthetic pathway (GltAB), conversion of glutamine to glutamate would be expected to decrease the intracellular glutamine level (Fig. 5B, panel b), and glnA expression would be derepressed by inactivation of the transcriptional factor GlnR in the absence of glutamine (19, 20). Collectively, the decreased expression of rocG in strain MGB874 resulted in activation of the genes encoding the GS-GOGAT pathway during stationary phase (Fig. 7).

Contribution of deletion of genes related to arginine and glutamate metabolism in strain MGB874 to cell yield and specific productivity. In MGB874 cells, the deletion of rocR induced considerable changes in metabolism and resulted in an increase in intracellular glutamate levels, which may have contributed to the improvement of cell yield. We further examined the contribution of each metabolic gene related to glutamate metabolism to cell yield and specific protein productivity by constructing several deletion mutants.

(i) Arginine metabolism. The deletion of ahrC, encoding the repressor of the argCJBD-carAB-argF operon, increased the expression level of argC in strain MGB874 to a level comparable to that in strain 874DEFR (Fig. 8A, panel b) and decreased cell yield and specific productivity (Fig. 8A, panel a). However, the additional deletion of the argCJBD-carAB-argF operon in the ahrC mutant of MGB874 recovered the reduced cell yield and specific productivity (Fig. 8A, panel a). Therefore, inhibition of the conversion of glutamate to arginine is essential for the improved cell yield in strain MGB874.

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Effects of glutamate feeding on cell yield and Egl-237 production. From the metabolic and transcriptomic analyses, the maintenance of the intracellular glutamate level resulting from deletion of the rocDEF-rocR region was strongly suggested to improve the cell yield and enhance Egl-237 production in strain MGB874. Therefore, we evaluated the effects of glutamate feeding on cell yield, Egl-237 production, and specific productivity in strains 168, MGB874, and 874DEFR. The feeding of sodium glutamate at a final concentration of 20 mM after depletion of glutamate in the culture broth led to increases in cell yields and Egl-237 production for all strains (see Fig. S3 in the supplemental material). However, glutamate feeding had little effect on the specific productivities (U g⁻¹ h⁻¹) and intracellular glutamate levels of the three strains (data not shown), indicating that intracellular glutamate concentrations are not directly proportional to cell yield. We also measured the extracellular pH under glutamate feeding con-
ditions and observed that the pH of the growth medium was not significantly influenced by glutamate feeding.

**DISCUSSION**

To our knowledge, *B. subtilis* strain MGB874 is the first reported reduced-genome strain to serve as an efficient host for the production of recombinant proteins. Here we demonstrated that the combined effects of improved cell yield and increased specific productivity enhanced recombinant enzyme production in MGB874 cells. Genetic analyses revealed that deletion of the *rocDEF-rocR* region in strain MGB874 was responsible for the improved cell yield and maintained the high specific productivity of reduced-genome strain MGB625 (Fig. 2).

The high specific productivities in the reduced-genome strains are likely attributable to the high transcriptional levels of egl-237 from multicopy plasmid pHYS237 compared to those in the wild-type strain, likely due to increases in the plasmid copy number and promoter activity of egl-237 (Fig. 3). These results imply at least two independent effects of genome reduction on specific productivity. Plasmid pHYS237 was derived from plasmid pUB110 (38), whose copy number in *B. subtilis* decreases in stationary phase compared with that in exponential phase (40). In addition, the egl-237 promoter contains the consensus sequence of a sigma A-type vegetative promoter (24). Previously, we reported that the activation of sporulation-specific sigma factors was delayed in MGB874 cells, suggesting that the transition state is extended in strain MGB874 compared to wild-type strain 168 (39). Therefore, although further experiments are needed, the increased plasmid copy number and promoter activity of egl-237 may be attributable to the prolonged maintenance of the vegetative state in MGB874 cells.

Deletion of the *rocDEF-rocR* region in strain MGB874 not only inactivated the arginine degradation pathway but also caused other considerable metabolic changes, including repression of the arginine synthetic pathway (*argCJBD-carAB-argF* operon) and glutamate dehydrogenase (*rocG*) and activation of aconitase (*citB*) and the GS-GOGAT pathway (*glnA* and *gltAB*) (Fig. 6 and 7). The drastic changes in nitrogen metabolism increased the intracellular glutamate level and may have contributed to the improved cell yield in strain MGB874. Glutamate is an important carbon source of 2-oxoglutarate and is the main nitrogen source for the synthesis of...
nearly all N-containing compounds (5). Additionally, glutamate serves as a counterbalance anion to the internal pool of K⁺ and is an essential cellular osmolyte (16). Yan et al. (35) reported that glutamate deficits result in a suboptimal K⁺ pool and in growth defects in Salmonella enterica serovar Typhimurium. However, we found that while the intracellular glutamate levels were not affected upon glutamate feeding, cell yields of the reduced-genome strains were increased, suggesting that intracellular glutamate levels as an anion pool are not proportional to increases in cell yield. Therefore, an increased supply of glutamate by enhanced glutamate metabolism is expected to improve the cell yield of strain MGB874. Glutamate is considered unnecessary as a carbon source in growth medium containing sufficient maltose. Thus, we favor the hypothesis that enhanced metabolism of glutamate leads to increased flux to the synthesis of other amino acids via transamination (5), resulting in enhanced cellular protein synthesis and corresponding increases in cell yield. Additionally, it is also possible that the increased glutamate supply modulates the stringent response, which is a stress response to amino acid starvation conditions and represses the proliferation of cells until nutrient conditions improve (17). Jung et al. (30) reported that the addition of glutamate to culture medium enhanced recombinant Thermus maltogenic amylase (THMA) production and increased the growth rate of E. coli by overcoming the stringent response. Therefore, enhanced glutamate metabolism might relax the stringent response, leading to the improvement of cell yields of strain MGB874.

Glutamate feeding significantly improved the cell yield of strain 874DEFR, indicating that a sufficient glutamate supply from the culture medium could preclude the positive effects of rocDEF-rocR deletion on cell yield and enzyme production. However, in practical terms, the deletion of rocDEF-rocR is a cost-effective strategy for improving enzyme production, because high enzyme yields were obtained using the relatively simple 2×L-Mal growth medium without supplementation.

For further enhancement of recombinant enzyme production in strain MGB874, it is necessary to improve three factors: transcript levels of the target gene, specific productivity, and cell yields. First, the increased plasmid copy numbers and promoter activities did not reflect the actual transcript levels of the target gene, specific productivity, and cell yields. In this case, the increased plasmid copy numbers and promoter activities did not result in the actual transcript levels of the target gene, specific productivity, and cell yields. However, in practical terms, the deletion of rocDEF-rocR is a cost-effective strategy for improving enzyme production, because high enzyme yields were obtained using the relatively simple 2×L-Mal growth medium without supplementation.

In conclusion, we revealed that two factors contribute to the high enzyme production levels in reduced-genome strain MGB874: increased specific productivity and improved cell yield. The combined effect of the improvement of cell yield by deletion of the rocDEF-rocR region and the increase in specific productivity by the deletion of other genomic regions enhances enzyme production in strain MGB874. We also found that the effect of rocDEF-rocR deletion on enzyme production is more pronounced in MGB874 cells than in wild-type 168 cells. Since full enzyme overproduction in strain MGB874 could not be achieved by single deletion of the rocDEF-rocR region, a multiple-deletion strategy may be necessary for the improved production of beneficial substances in Bacillus subtilis. Future research to improve cell yield and increase specific productivity is expected to lead to further enhancement of Egl-237 production in strain MGB874.

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