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RESEARCH ARTICLE

Combined Drug Resistance Mutations Substantially Enhance Enzyme Production in *Paenibacillus agaridevorans*

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ABSTRACT This study shows that sequential introduction of drug resistance mutations substantially increased enzyme production in *Paenibacillus agaridevorans*. The triple mutant YT478 (*rsmG* Gln225→stop codon, *rpsL* K56R, and *rpoB* R485H), generated by screening for resistance to streptomycin and rifampin, expressed a 1,100-fold-larger amount of the extracellular enzyme cycloisomaltooligosaccharide glucanotransferase (CITase) than the wild-type strain. These mutants were characterized by higher intracellular *S*-adenosylmethionine concentrations during exponential phase and enhanced protein synthesis activity during stationary phase. Surprisingly, the maximal expression of CITase mRNA was similar in the wild-type and triple mutant strains, but the mutant showed greater CITase mRNA expression throughout the growth curve, resulting in enzyme overproduction. A metabolome analysis showed that the triple mutant YT478 had higher levels of nucleic acids and glycolysis metabolites than the wild type, indicating that YT478 mutant cells were activated. The production of CITase by the triple mutant was further enhanced by introducing a mutation conferring resistance to the rare earth element, scandium. This combined drug resistance mutation method also effectively enhanced the production of amylases, proteases, and agarases by *P. agaridevorans* and *Streptomyces coelicolor*. This method also activated the silent or weak expression of the *P. agaridevorans* CITase gene, as shown by comparisons of the CITase gene loci of *P. agaridevorans* T-3040 and another cycloisomaltooligosaccharide-producing bacterium, *Paenibacillus* sp. strain 598K. The simplicity and wide applicability of this method should facilitate not only industrial enzyme production but also the identification of dormant enzymes by activating the expression of silent or weakly expressed genes.

IMPORTANCE Enzyme use has become more widespread in industry. This study evaluated the molecular basis and effectiveness of ribosome engineering in markedly enhancing enzyme production (>1,000-fold). This method, due to its simplicity, wide applicability, and scalability for large-scale production, should facilitate not only industrial enzyme production but also the identification of novel enzymes, because microorganisms contain many silent or weakly expressed genes which encode novel antibiotics or enzymes. Furthermore, this study provides a new mechanism for strain improvement, with a consistent rather than transient high expression of the key gene(s) involved in enzyme production.

KEYWORDS enzyme production, silent genes, ribosome engineering, drug resistance, metabolome analysis

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At present, industrially produced enzymes are used in a broad variety of production processes, including the production of pulp and paper (e.g., cellulase, xylanase, and laccase), leather (e.g., protease and lipase), textiles (e.g., catalase and arylesterase), detergents (e.g., protease, cellulase, and lipase), food and beverages (e.g., amylase, protease, lipase, and pectinase), animal feed (e.g., xylanase, protease, and phytase), pharmaceuticals (e.g., lipolase and D-amino acid oxidase), fine chemicals (e.g., lipase), cosmetics (e.g., lipase), and biodiesel (e.g., lipase) (1). This has increased interest in methods that enhance enzyme production and activity (2–4). Classically, enzyme production by microorganisms has been enhanced by random mutagenesis, including mutants induced by chemicals such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS) and by UV radiation (5, 6). Strain improvement processes have been described for producing various industrial enzymes, including lipases, chitinases, cellulases, glucoamylases, and proteases (7–12). Enzymes for industrial use are produced by growing bacteria and fungi under submerged or solid-state conditions. In practice, the great majority of microbial enzymes come from a very limited number of genera, especially *Aspergillus*, *Trichoderma*, *Bacillus*, and *Kluyveromyces*. Most of the strains used by the food industry have either been employed for many years or were derived from such strains by mutation and selection (13). However, these approaches require much time, cost, and labor.

Genome shuffling is an alternative that enables the directed evolution of entire organisms via recursive recombination at the genome level, thus shortening the time required for strain improvement (14, 15). Unlike mutagenesis techniques, genetic engineering methods, such as gene cloning and the transformation of host organisms, are often effective in improving enzyme production, although genetic information is required beforehand (16, 17). The industrial production of recombinant enzymes is preceded by an extensive research and development phase that culminates in the construction of an optimal strain for enzyme production (16). The desired industrial goal, however, is often difficult to achieve using the native form of the enzyme. Recent developments in protein engineering have revolutionized the transformation of commercially available enzymes to better industrial catalysts. Protein engineering aims to modify the sequence of a protein, and hence its structure, to create enzymes with improved functional properties, such as stability, specific activity, inhibition by reaction products, and selectivity toward nonnatural substrates (17).

We previously demonstrated that modulating the cellular translational and transcriptional apparatus, by introducing spontaneous drug resistance mutations (ribosome engineering), is effective in activating silent or weakly expressed secondary metabolite genes, enabling the discovery of novel antibiotics (18–22). The concept of ribosome engineering was derived from findings using a *Streptomyces lividans* strain with an altered ribosomal S12 protein that confers resistance to streptomycin (23). This strain produced abundant quantities of the blue-pigmented antibiotic actinorhodin, which is not normally produced by *S. lividans* due to the dormancy of the antibiotic biosynthesis genes (23). Furthermore, the bacterial alarmone ppGpp, produced on the ribosome in response to nutrient starvation, was found to bind to RNA polymerase (24–26), eventually initiating the production of antibiotics (19, 27, 28). These results suggested that modified RNA polymerase, produced by introducing a rifampin resistance mutation, may mimic the ppGpp-bound form, activating the expression of biosynthetic gene clusters (29, 30).

We hypothesized that bacterial gene expression may be increased dramatically by altering transcription and translation pathways. That is, a ribosome engineering method targeting the ribosomal protein S12, other ribosomal proteins, and translation factors or RNA polymerases may activate or enhance the production of secondary metabolites. Using this method, strains with mutations in the genes *rsmG* (encoding the 16S rRNA methyltransferase), *rpsL* (encoding the ribosomal protein S12), and *rpoB* (encoding the RNA polymerase β-subunit) were isolated by selection with streptomycin and rifampin. Cumulative drug resistance mutations (e.g., octuple mutations) were

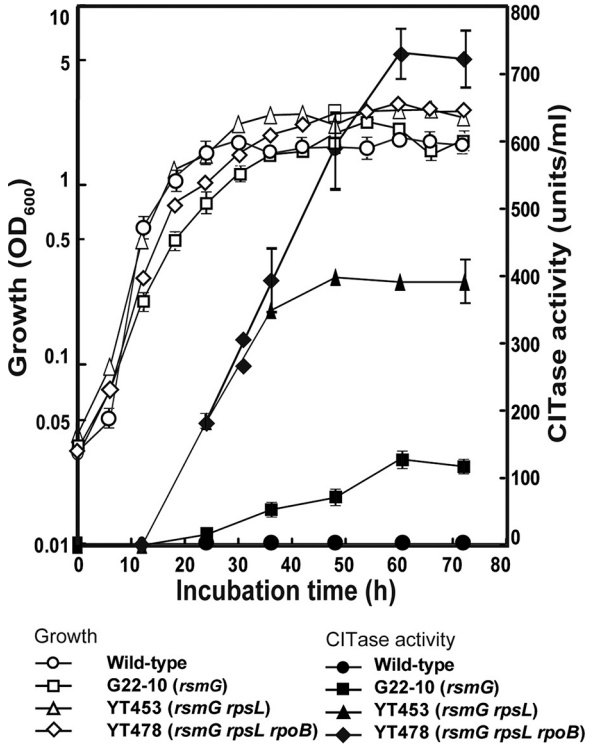

FIG 1 Growth and CITase production by wild-type strain T-3040, mutant strain G22-10, double mutant strain YT453, and triple mutant strain YT478. Strains were grown in liquid LB-Dex medium. Culture samples were withdrawn at the indicated times, and cell densities (growth) and CITase activities were measured. One unit of CITase activity was defined as the amount of enzyme that produced 1 nmol of the sum of CI-7, CI-8, and CI-9 per min from dextran at pH 5.5 at 40°C. Each strain was cultured in six culture flasks, and the results are expressed as means ± standard deviations.

found effective in markedly enhancing antibiotic production in *Streptomyces* species (31, 32). These methods do not require genetic information. This study reports that ribosome engineering can enhance enzyme production, using as an example cycloisomaltooligosaccharide glucanotransferase (CITase; EC 2.4.1.248) of *Paenibacillus agaridevorans*, an extracellular enzyme that catalyzes the formation of cycloisomaltooligosaccharides (CIs; cyclodextrans) from dextran and is utilized by the food industry as a masking agent for bitter substances.

RESULTS

Isolation and characterization of drug-resistant mutants. *P. agaridevorans* wild-type strain T-3040 (formerly *Bacillus circulans* T-3040) produces little CITase (≤1 unit), suggesting that the gene encoding this enzyme is silent or weakly expressed (Fig. 1). The introduction of a spontaneous drug resistance mutation (*rsmG*, *rpsL*, or *rpoB*) by selecting for resistance to streptomycin or rifampin, thereby activating silent genes involved in the synthesis of secondary metabolites in actinomycetes, enhanced the production of CITase up to 215-fold (Table 1). Notably, the *rsmG* mutants, many of which resulted in frameshifts or the introduction of a stop codon, displayed similar resistance levels to streptomycin and the constitutive ability to produce 90- to 113-fold more CITase than T-3040, whereas the *rpsL* and *rpoB* mutants showed various levels of CITase production. Mutations in position Gln469 of *rpoB* resulted in the production of 174- to 215-fold more CITase than from T-3040. CITase has been produced industrially in Japan using the *P. agaridevorans* strain G22-10, which was derived from strain T-3040 by treatment with NTG and selecting for low-level resistance to streptomycin (33). As expected, strain G22-10 harbored a mutation (673C→T [Gln225→stop codon]) within the *rsmG* gene (Table 2), with high CITase productivity, equivalent to that of spontaneous *rsmG* mutants (Table 1).

TABLE 1 Characterization of streptomycin- and rifampin-resistant mutants of *P. agaridevorans* T-3040

Strain	Antibiotic (μg/ml) used for selection	Mutation[a]			Amino acid substitution	Resistance (μg/ml)[b]		CITase activity (U/ml)[c]
		rsmG	*rpsL*	*rpoB*		Streptomycin	Rifampin	
T-3040 (wild type)						20	0.5	≥1
YT362	Streptomycin (30)	118–121→CGAA→Δ			Frameshift (position 40→stop codon)	150	0.5	90 ± 41
YT359	Streptomycin (30)	119G→1-bp deletion			Frameshift	150	0.5	109 ± 13
YT361	Streptomycin (30)	148G→AA			Frameshift	150	0.5	96 ± 10
YT360	Streptomycin (30)	544G→T			Glu182→stop codon	150	0.5	107 ± 11
YT373	Streptomycin (30)	685C→T			Arg229→Cys	150	0.5	113 ± 16
YT382	Streptomycin (100)		167A→G		Lys56→Arg	3000	0.5	140 ± 38
YT374	Streptomycin (100)		192G→T		Lys64→Asn	50	0.5	114 ± 8
YT420	Streptomycin (100)		301A→G		Lys101→Glu	1000	0.5	35 ± 16
YT421	Streptomycin (100)		302A→G		Lys101→Arg	500	0.5	99 ± 11
YT385	Streptomycin (100)		302A→T		Lys101→Met	500	0.5	109 ± 16
YT386	Streptomycin (100)		314G→A		Gly105→Asp	200	0.5	72 ± 12
YT504	Rifampin (2)			1393T→C	Ser465→Pro	20	5	32 ± 18
YT510	Rifampin (2)			1405C→A	Gln469→Lys	20	300	197 ± 62
YT399	Rifampin (2)			1406A→T	Gln469→Leu	20	30	215 ± 67
YT393	Rifampin (2)			1406A→G	Gln469→Arg	20	300	174 ± 3
YT506	Rifampin (2)			1415A→T	Asp472→Leu	20	2	3 ± 20
YT395	Rifampin (2)			1433C→A	Ala478→Asp	20	30	59 ± 12
YT398	Rifampin (2)			1460C→A	Ser487→Thr	20	30	110 ± 25
YT394	Rifampin (2)			1460C→T	Ser487→Phe	20	30	80 ± 8

[a]Numbered from the start codon of the open reading frame of *B. subtilis*.

[b]Determined after incubating for 3 days on NG plates.

[c]Strains were grown in LB-Dex medium at 30°C for 3 days. One unit of CITase was defined as the amount of enzyme capable of producing 1 nmol of the sum of CI-7, CI-8, and CI-9 per min from dextran at pH 5.5 at 40°C. Experiments were performed in triplicate, and the data are expressed as means ± standard deviations.

TABLE 2 Sequential introduction of streptomycin and rifampin resistance mutations into the *P. agaridevorans rsmG* mutant G22-10

Strain[a]	Antibiotic (μg/ml) used for selection	Mutation[b]			Amino acid substitution	Resistance (μg/ml)[c]		CITase activity (U/ml)[d]
		rsmG	*rpsL*	*rpoB*		Streptomycin	Rifampin	
G22-10[d]		673C→T			Gln225→stop codon	300	0.5	117 ± 8
YT453	Streptomycin (2,000)	673C→T	167A→G		Lys56→Arg	3,000	0.5	518 ± 77
YT483	Rifampin (2)	673C→T	167A→G	1406A→G	Gln469→Arg	3,000	300	168 ± 142
YT474	Rifampin (2)	673C→T	167A→G	1433C→A	Ala478→Asp	3,000	30	432 ± 72
YT477	Rifampin (2)	673C→T	167A→G	1444C→G	His482→Asp	3,000	100	644 ± 45
YT502	Rifampin (2)	673C→T	167A→G	1444C→T	His482→Tyr	3,000	100	195 ± 42
YT503	Rifampin (2)	673C→T	167A→G	1445C→G	His482→Arg	3,000	100	112 ± 99
YT478	Rifampin (2)	673C→T	167A→G	1454G→A	Arg485→His	3,000	30	1,104 ± 143
YT475	Rifampin (2)	673C→T	167A→G	1460C→T	Ser487→Phe	3,000	30	605 ± 66

[a]G22-10 is the parent strain, from which YT453 was derived. YT483 to YT475 were derived from YT453.
[b]Numbered from the start codon of the open reading frame of *B. subtilis*.
[c]Determined after incubation for 3 days on NG plates.
[d]Strains were grown in LB-Dex medium at 30°C for 3 days. One unit of CITase was defined as the amount of enzyme capable of producing 1 nmol of the sum of CI-7, CI-8, and CI-9 per min from dextran at pH 5.5 at 40°C. Experiments were performed in triplicate, and the data are expressed as means ± standard deviations.

Construction of combined drug-resistant mutants. Starting from the industrial strain G22-10 (*rsmG*), we attempted to further increase CITase production by introducing cumulative drug resistance mutations. The introduction of an *rpsL* mutation (Lys56→Arg) by selecting for high-level resistance to streptomycin increased CITase production 4.4-fold. CITase production was further increased by introducing a third mutation, *rpoB* (Arg485→His), which confers resistance to rifampin, with the triple mutant YT478 producing 1,104 units/ml CITase, more than 1,000-fold higher than that of the wild-type strain T-3040 (Fig. 2A and Table 2). This amount corresponds to approximately 100 mg CITase per liter (or 0.01%), as determined by protein assay of culture supernatants. The triple mutant strain grew as well as the wild-type strain and produced CITase even after entering into stationary-growth phase (Fig. 1). Unexpectedly, several *rpoB* mutations (Gln469→Arg and Ala478→Asp), which enhanced CITase production when introduced into the wild-type strain, did not enhance CITase production when introduced into the double mutant (Table 2). These findings suggested that only a limited number of *rpoB* mutations can have positive effects in improved strains, such as the double mutant YT453, because improved strains with multiple mutations would become more sensitive metabolically. This mechanism may also explain the

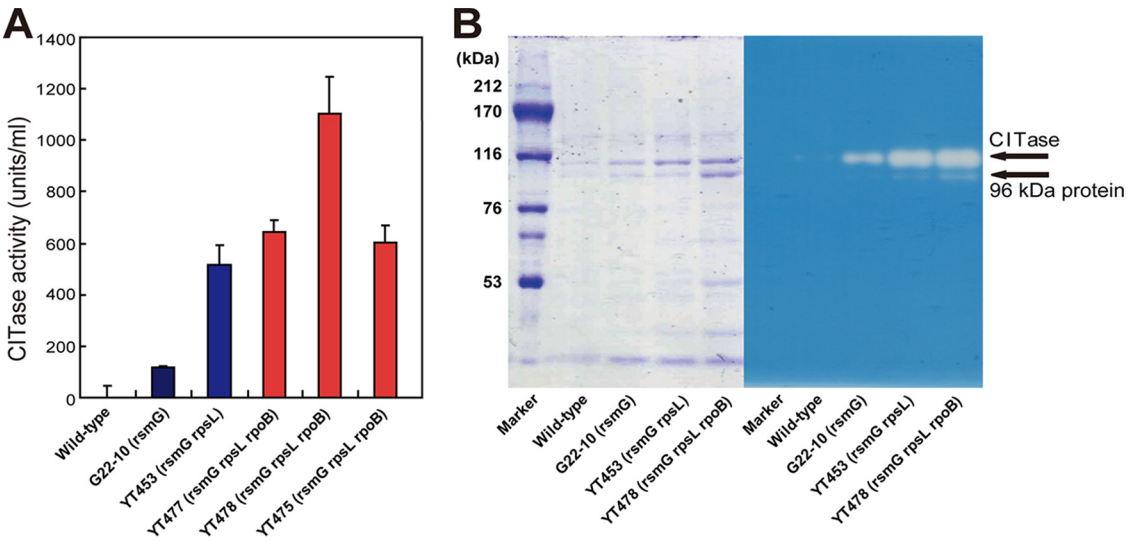

FIG 2 Detection and analysis of CITase. (A) CITase production by wild-type and single, double, and triple mutant strains. One unit of CITase was defined as the amount of enzyme that produced 1 nmol of the sum of CI-7, CI-8, and CI-9 per min from dextran at pH 5.5 at 40°C. Experiments were performed in triplicate, and the results are expressed as means ± standard deviations. (B) SDS-PAGE (left) and zymography (right) of CITase. The arrows indicate native CITase and the 96 kDa protein.

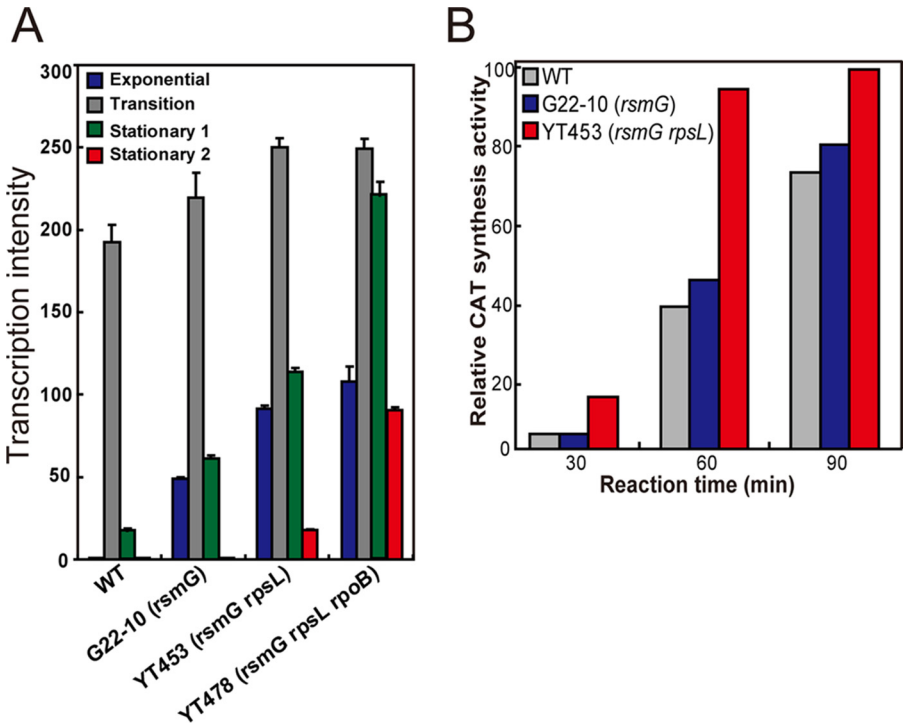

FIG 3 Functional analysis of mutant strains. (A) Transcriptional analysis of the gene encoding CITase. Strains were grown in LB-Dex medium at 30°C to exponential phase (OD_{600} of 0.2), transition phase (OD_{600} of 0.7), early stationary phase (12 h after transition phase), and late stationary phase (24 h after transition phase). (B) *In vitro* protein synthesis activities of mutant strains as determined by chloramphenicol acetyltransferase (CAT) activity. Strains were grown to late exponential phase (OD_{600} of 0.4), and cell-free synthesis of CAT by washed ribosomes was performed as described in Materials and Methods. Experiments were performed in triplicate, and the results are expressed as means ± standard deviations.

reduced CITase productivity of YT502 and YT503. The CITase production profiles of the wild-type and mutant strains were confirmed by SDS-PAGE and zymography (Fig. 2B). The molecular size of CITase was predicted to be 103 kDa, similar to that of the protein band on Coomassie brilliant blue R-250 (CBB)-stained gels and the halos observed by zymography. A smaller-sized band detected at 96 kDa with reduced enzyme activity may be the C-terminal-truncated form of CITase, which was partially digested by proteases secreted into the medium (33). Notably, dextran, but not other carbohydrates, induced CITase production by wild-type (T-3040) and mutant (YT453 and YT478) strains (see Fig. S1 in the supplemental material).

Transcriptional analysis of the mutants. Transcriptional analysis was performed to assess the mechanism underlying CITase overproduction. The expression of CITase mRNA was transient in the wild-type strain T-3040, being expressed only during transition phase (Fig. 3A). Strikingly, its periods of expression were greater in single (G22-10), double (YT453), and triple (YT478) mutant strains, in that order, although their maximum expression levels did not differ markedly, accounting for the constitutive production of CITase by YT453 and YT478 (Fig. 1). This contrasts with secondary metabolites, in that the enhanced antibiotic production by drug resistance mutants was almost always accompanied by much higher maximal expression levels of antibiotic biosynthesis genes (20, 21, 31, 32). A primer extension analysis of *cit* (see Fig. S2) confirmed these results, by demonstrating the absence of additional mutations as well as by detecting the transcriptional start points, at −10 and −35 hexamers, and the ribosome binding site (Shine-Dalgarno [SD] sequence). However, codons 2 (GTG) and 5 (ATG) may be potential start codons, as GTG is often used as a translation start codon (Fig. 4A and B).

Translational analysis of the mutants. We next studied the protein synthesis activity and intracellular *S*-adenosylmethionine (SAM) levels of mutant and wild-type

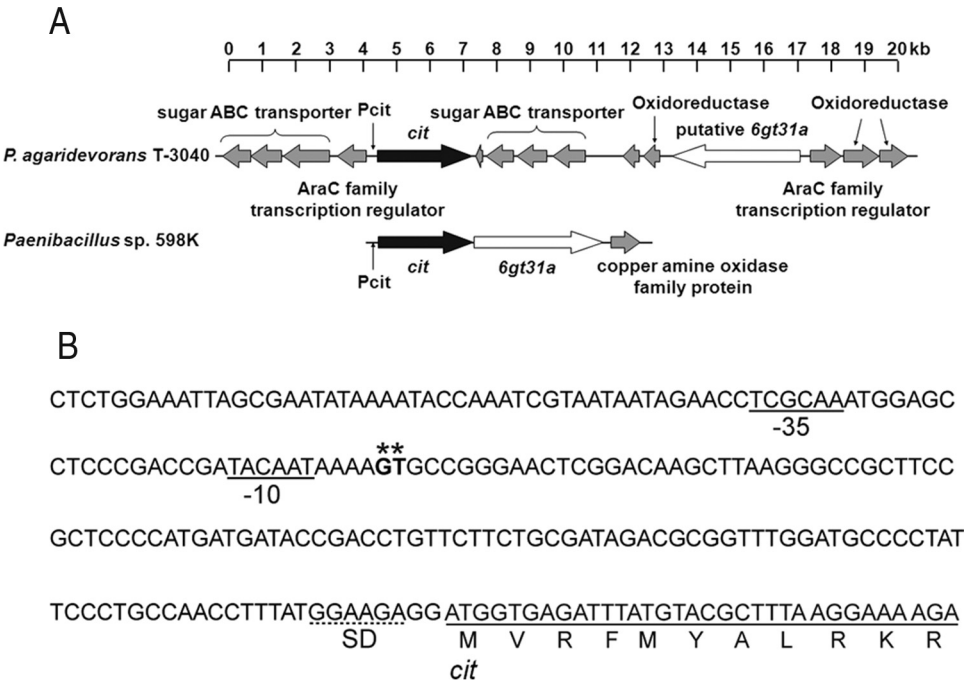

FIG 4 Molecular analysis of the CITase gene. (A) Schematic representation of gene loci containing *cit* in the CI-producing bacteria. The gene loci (contig_0325 [PAT3040_05184 to PAT3040_05199]) of *P. agaridevorans* T-3040 (top) and *Paenibacillus* sp. 598K (GenBank accession no. LC160266.1 [bottom]) are indicated. Genes encoding CITase (*cit*), 6-α-glucosyltransferase (*6gt31a*), and other proteins are shown as black, white, and gray arrows, respectively. Pcit, promoter region of the *cit* gene. (B) Promoter region of the *cit* gene. The transcriptional start points for the *cit* gene are indicated by asterisks. The −10 and −35 hexamers are underlined, and the ribosome binding site (SD sequence) is indicated by a dotted underline.

strains. To avoid the possible effects of *rpoB* and/or *rpsL* mutations, the single mutant YT362 (*rsmG*) and the double mutant YT453 (*rsmG rpsL*), rather than the triple mutant YT478, were compared with the wild type. The *rsmG rpsL* double mutant YT453 was found to have a 2.5-fold higher *in vitro* protein synthesis activity than the wild type, an increase apparently due to the *rpsL* mutation, as determined by chloramphenicol acetyltransferase activity (Fig. 3B; see also Fig. S3). In addition, the expression of the *metK* gene, which encodes the enzyme SAM synthetase, was significantly enhanced in the *rsmG* mutant YT362 (and in other *rsmG* mutants G22-10, YT453, and YT478) (Fig. 5), resulting in higher intracellular SAM levels (see Fig. S4). These results are consistent

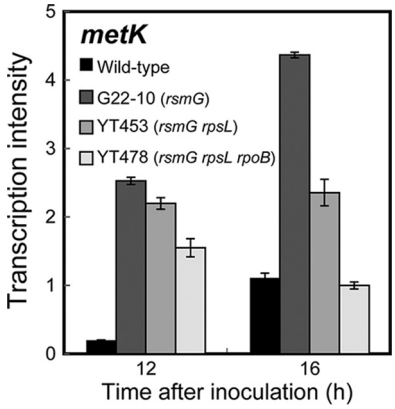

FIG 5 Transcriptional analysis of the *metK* gene encoding *S*-adenosylmethionine synthetase in the wild-type and mutant (G22-10, YT453, and YT478) strains harboring the *rsmG* mutation. Transcription of *metK* was determined as described in Materials and Methods. Experiments were performed in triplicate, and the results are expressed as means ± standard deviations.

TABLE 3 Intracellular concentrations of key metabolites during exponential (12 h) growth of the wild-type (T-3040) and triple mutant (YT478) strains[a]

Metabolite	Concn (pmol/OD unit)[b]	
	Wild-type (T-3040)	Mutant (YT478)
SAM[c]	134 ± 11	274 ± 19
ATP	970 ± 45	1,431 ± 330
GTP	159 ± 45	304 ± 158
UTP	247 ± 9.7	457 ± 115
CTP	234	756 ± 71
Glucose 6-phosphate	207 ± 167	527 ± 229
Fructose 1,6-diphosphate	185 ± 147	1,000 ± 572
3-Phosphoglyceric acid	465 ± 226	1,565 ± 406
2-Phosphoglyceric acid	68 ± 42	157 ± 24
Phosphoenolpyruvic acid	133 ± 59	446 ± 77

[a]Strains were grown to mid-exponential (12 h) phase in LB-Dex medium. Intracellular metabolites were extracted and subjected to metabolome analysis.
[b]All experiments were performed in triplicate, and the data are expressed as means ± standard deviations.
[c]SAM, *S*-adenosylmethionine.

with findings showing that, in *Streptomyces* and *Bacillus*, protein synthesis activity during transition and stationary phases and intracellular SAM levels are both crucial for the activation or enhancement of secondary metabolism, including antibiotic production, as demonstrated by *rpsL*, *rsmG*, and *mthA* mutants (18, 34–37).

Metabolome analysis of the mutants. Metabolome analysis was performed to assess the metabolic changes resulting from the triple mutations in *rsmG*, *rpsL*, and *rpoB*. A comparison of the triple mutant YT478 with wild-type strain T-3040 showed many differences in metabolism (see Data Set S1). YT478 had higher levels of nucleic acids and glycolysis metabolites during mid-exponential phase. This was especially pronounced for the nucleoside triphosphates (ATP, GTP, UTP, and CTP), the concentrations of which were 1.5- to 3-fold higher in YT478 than in the wild type. The differences in key metabolites between wild-type and triple mutant strains are summarized in Table 3. Notably, the level of intracellular SAM during mid-exponential phase (12 h) was 2-fold higher in the triple mutant than in the wild type (Table 3), consistent with the increased expression of *metK* in YT478 (Fig. 5) and suggesting the significance of the SAM level in activating enzyme production. These metabolic changes may be a reflection of the "activated" YT478 mutant cells and may play at least a partial role in the overproduction of CITase. For example, the elevated concentrations of nucleoside triphosphates, especially ATP and GTP, may contribute to the cellular energy status required for CITase overproduction. Moreover, many metabolites, including pyruvic acid, 2-oxoglutaric acid, citric acid, gluconic acid, ribulose 5-phosphate, sedoheptulose 7-phosphate, GMP, guanosine, inosine, and hypoxanthine, were significantly enriched at 48 h in the wild type but not in YT478. Although the association of these events with CITase productivity remains unclear, marked metabolic differences were observed between the wild-type and triple mutant strains. The lower levels of almost all amino acids observed at 48 h in the triple mutant YT478 may be explained by the active CITase production at this growth phase, which would consume intracellular amino acids.

Further enhancement of CITase production by scandium resistance mutations. We previously reported that certain mutations conferring resistance to rare earth elements such as scandium effectively increase antibiotic production by *Streptomyces* spp. (19) and amylase production by *Bacillus subtilis* (38). As expected, the introduction of certain scandium resistance mutations further increased CITase production. More than half of the 18 scandium-resistant mutants derived from the triple mutant YT478 showed higher production of CITase than the parental strain YT478. The representative strain SC9, which was resistant up to 500 μg/ml of $ScCl_3 \cdot 6H_2O$, displayed a 1.4-fold-greater production of CITase than YT478, although the mutated gene has not yet been identified. These results show that scandium resistance mutations not only enhance secondary metabolism (as represented by antibiotic production) but enhance the production of certain extracellular enzymes.

Gene locus for CI biosynthesis. To compare the CITase gene *cit* in *P. agaridevorans* with that of another CI-producing bacterium, *Paenibacillus* sp. strain 598K, we sequenced the genome of the wild-type strain T-3040. The draft genome of T-3040 contained 467 contigs with a total length of 8,694,911 bp and a coverage depth of 108×. The largest contig was 597,596 bp, the N_{50} contig length was 117,306 bp, and the genome contained 6,887 open reading frames (ORFs). A genome analysis revealed that this strain possesses only one gene encoding CITase (locus tag PAT3040_05188). According to the CAZy database (39), the CITase in T-3040 belongs to the glycoside hydrolase family 66 (GH66), which consists of CITases and dextranases (EC 3.2.1.11). As reported previously (40), a gene in the AraC family encoding a regulatory protein (PAT3040_05187) was found upstream of *cit*, and genes encoding putative sugar ABC transporters were located on the opposite strand of *cit* both upstream (PAT3040_05184 to PAT3040_05186) and downstream (PAT3040_05190 to PAT3040_05192) (Fig. 4A). A gene (putative *6gt31a* [PAT3040_05195 and PAT3040_05196]) encoding a putative glycoside hydrolase with approximately 60% amino acid sequence identity to *Paenibacillus* sp. 598K 6-α-glucosyltransferase, which is essential for the production of the substrate of CITase from starch (40), was found 7,625 bp downstream from *cit* on the opposite strand. Moreover, a distinct AraC regulator gene (PAT3040_05197) was found upstream of the putative *6gt31a*. Importantly, the draft genome contained no potential dextranase genes other than *cit* (see Table S2).

Industrial applicability of the triple mutant YT478. The productivity of microbial metabolites is often markedly decreased under conditions of large-scale production. This may be due, for example, to mechanical reasons in fermentation and/or the genetic instability of the microorganism. We therefore assessed the productivity of CITase by *P. agaridevorans* by using a 90-liter jar fermentor and a 300-liter tank. The levels of CITase produced by the triple mutant YT478 were as high under large-scale conditions as under laboratory conditions (97% in the 90-liter jar fermentor and 78% in the 300-liter tank compared with that in shake flask fermentation). Moreover, the CITase productivity of YT478 was 6- to 10-fold higher than that of the industrial strain G22-10 under large-scale conditions (for example, 802 units/ml [YT478] versus 110 units/ml [G22-10] in 90-liter jar fermentors). These results indicate that the YT478 strain may be used for industrial applications.

Applicability for production of other enzymes. The introduction into *B. subtilis* of certain *rpsL* and *rpoB* mutations has been reported to enhance the production of amylases and proteases 1.3- to 3.5-fold (41, 42). Similarly, the introduction of the *rpsL* K88R mutation into a marine-derived strain of *Streptomyces viridochromogenes* was found to increase xylanase production 14% (43). The combined drug resistance mutation method was found to be widely applicable to enhance the production of certain extracellular enzymes. For example, *P. agaridevorans* production of amylase was enhanced 4.3-fold by introducing the *rsmG*, *rpsL*, and *rpoB* mutations (Fig. 6A). The introduction of certain *rsmG*, *rpsL*, and *rpoB* mutations into *Streptomyces coelicolor* enhanced agarase production 6.3-fold, but had a less pronounced effect on protease production, as shown by a 2.2-fold increase (Fig. 6B; see also Fig. S5). To normalize productivity, the production of these enzymes per numbers of cells, as assessed by optical density at 600 nm (OD_{600}) or mg dry cell weight, was also determined (see Fig. S6). Unlike dormant or weakly expressed enzymes (such as CITase), a >3-fold increase in the productivity of any highly expressed enzyme (including amylase, protease, and agarase) is not trivial, as the improvement of high-production strains has been reported to be difficult.

DISCUSSION

Microbial enzymes are of great importance in the development of industrial bioprocesses. The global market for these enzymes is continuing to grow and was estimated to be $5 billion in 2015 (13). Because enzymes for industrial use must be produced at low cost, many techniques have been developed for strain improvement (5, 6, 14–16). Currently, CIs are commercially produced from dextran using CITase

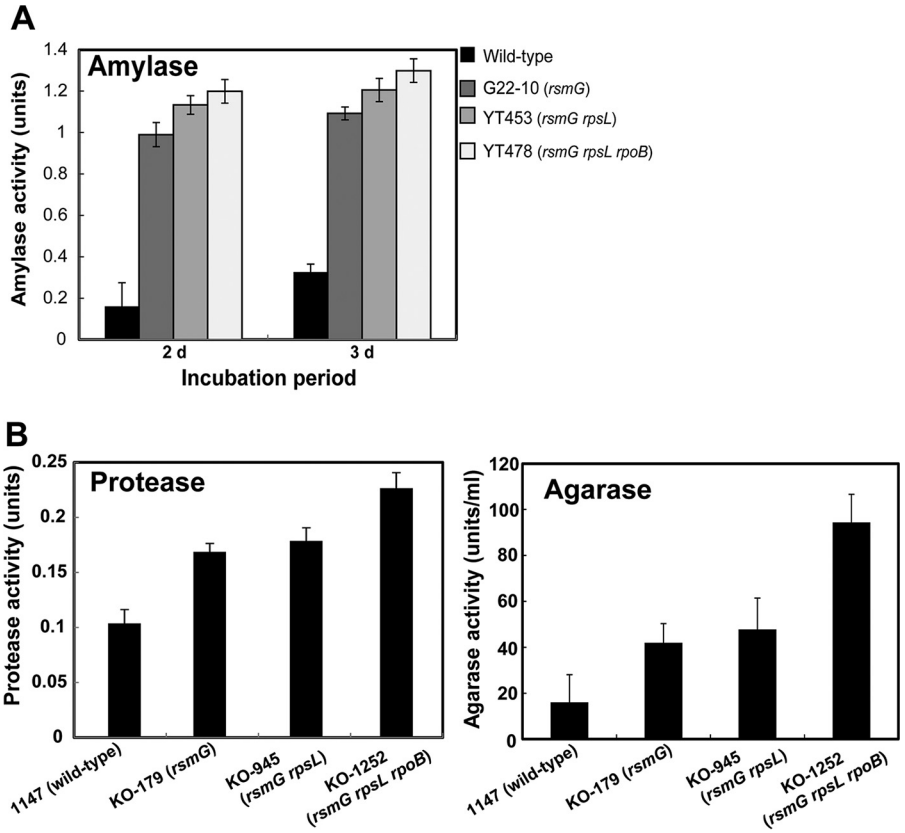

FIG 6 Enzyme production by the wild-type and mutant strains of *P. agaridevorans* and *S. coelicolor*. (A) Production of amylase by *P. agaridevorans* wild-type and single, double, and triple mutant strains. Strains were grown at 30°C for the indicated periods in LB medium supplemented with 1.5% glucose and 1% soluble starch. (B) Production of protease and agarase by *Streptomyces coelicolor* A3(2) and its mutant strains. Strains were grown at 30°C for 2 days in liquid skim milk medium (0.5% skim milk in deionized water) to measure protease production (left) or for 3 days in AYM medium, consisting of GYM medium (75) with 0.4% glucose replaced by 1% sodium aspartate, supplemented with 0.1% agarose to measure agarase production (right). Experiments were performed in triplicate, and the results are expressed as means ± standard deviations.

obtained by culturing *P. agaridevorans* G22-10. We have shown that certain mutations that alter the ribosome (i.e., *rsmG* and *rpsL* mutations) and RNA polymerase β-subunit (i.e., *rpoB* mutations) cumulatively enhance enzyme production. Apart from such technical aspects, this study provides a new mechanism for strain improvement, resulting in constitutive, rather than transient, high expression of the key gene(s) involved in enzyme production. Similar findings have been observed in assessing antibiotic production by *B. subtilis*, in that strains carrying certain drug resistance (*mthA*) mutants associated with bacilysin overproduction expressed bacilysin biosynthesis genes throughout all growth phases (37, 44).

Strikingly, we found that the *rpsL* K56R mutation in the ribosomal protein S12 of *P. agaridevorans*, similar to the *rpsL* K56R mutation of *B. subtilis* and the *rpsL* K87E mutation of *Escherichia coli* (41, 45), resulted in enhanced protein synthesis activity during late exponential phase (Fig. 3B). Certain mutations within S12 activate or enhance antibiotic production in a variety of bacteria, and the *rpsL* K88E mutation (corresponding to position 87 in *E. coli*) enhanced the protein synthesis activity of *S. coelicolor* during transition and stationary phases, resulting in the overproduction of the blue-pigmented polyketide antibiotic actinorhodin (36, 46). In *Pseudomonas putida*, the *rpsL* K88R mutation conferred enhanced tolerance to toxic organic chemicals (47). The mechanisms underlying the generation of these mutants have been studied extensively in *Streptomyces* and *Bacillus*. The streptomycin-resistant (Sm^r) *rpsL* mutant

ribosomes, carrying an amino acid substitution in ribosomal protein S12 that confers a high level of resistance to streptomycin, are more stable than wild-type ribosomes under stress conditions, such as amino acid starvation and low magnesium concentration, indicating that increased stability may enhance protein synthesis in stationary phase (35, 41). This characteristic may result in the enhancement during stationary phase of protein production from newly transcribed genes, such as those involved in antibiotic production. These findings suggest that both the greater stability of 70S ribosomes and the elevated levels of ribosome recycling factor resulting from the K88E mutation are responsible for enhanced protein synthesis in stationary phase, with the latter being responsible for antibiotic overproduction by the K88E mutant (35). The K88E mutation in S12, as well as *rsmG* mutations, result in the preferential transcription during stationary phase of *S. coelicolor* of specific genes, such as pathway-specific regulatory genes (*actII*-ORF4 and *redD*) governing antibiotic production (31, 36, 48). Although the mechanism by which the *rpsL* K88E mutation mediates such preferential gene transcription remains unclear, higher-order regulatory proteins may govern the expression of these pathway-specific regulatory genes.

Unlike *rpsL* mutations, mutations in *rsmG* (encoding 16S rRNA methyltransferase) have been shown to cause low-level streptomycin resistance in *E. coli*, *Mycobacterium tuberculosis*, *S. coelicolor*, and *B. subtilis* (32, 36, 49, 50). The loss of the m^7G modification in 16S rRNA results in resistance to streptomycin, providing a molecular basis for *rsmG* mutation-induced streptomycin resistance. Our finding that SAM may play an essential role in triggering the onset of enzyme production is not unprecedented. For example, *rsmG* mutants in *S. coelicolor* exhibit enhanced expression of the *metK* gene encoding SAM synthetase, accompanied by increased protein synthesis during stationary phase and eventually leading to the overproduction of antibiotics (36). Thus, increases in SAM synthetase (i.e., increased SAM levels) resulting from *rsmG* mutations likely activated secondary metabolism in *S. coelicolor* (32, 36). Furthermore, the addition of SAM resulted in the overproduction of antibiotics by streptomycetes (51, 52), indicating that SAM may be a common intracellular signal molecule for the onset of secondary metabolism in *Streptomyces* and *Bacillus* (34, 36, 37). The *rpsL* K88E mutant did not show any enhancement of SAM synthetase expression (T. Hosaka and K. Ochi, unpublished data). The physiological significance of SAM has been discussed in *B. subtilis* and *Streptomyces* spp. (53–55). SAM is synthesized from methionine and ATP by a SAM synthetase encoded by the *metK* gene and plays a central role in many cellular functions (56–58). In contrast to *Streptomyces* cells (34), extracellular SAM cannot be incorporated by *E. coli* or *B. subtilis* cells (59, 60). Intracellular SAM levels are likely tightly regulated in native *B. subtilis* cells, as a 2-fold increase was found to markedly alter cellular physiology and morphology (37). SAM is the methyl donor for the methylation of cytosine and adenosine bases in DNA, rRNA, and tRNA, various proteins, and small molecules important for both lower and higher organisms (56, 61, 62). In *E. coli*, SAM was shown to be a corepressor of the methionine regulation system (57). Although there is no evidence to date for the existence of a methyltransferase involved in the regulation of antibiotic or enzyme production, SAM-dependent protein methylation may play a role in controlling the activity of regulatory proteins that control the expression of antibiotic biosynthesis or enzyme-encoding genes. Alternatively, DNA or RNA methylation may be involved in regulating the expression of these genes (63, 64).

In contrast to *rpsL* and *rsmG* mutations, certain rifampin-resistant (Rif^r) *rpoB* mutations, which yield RNA polymerases with mutant β-subunits, activate antibiotic production by increasing the affinities of mutant RNA polymerases for promoters of certain genes in *S. coelicolor*, eventually enhancing the transcription of antibiotic biosynthesis genes (18). A subset of rifampin resistance (*rpoB*) mutations was found to result in the marked overproduction of antibiotics in various actinomycetes, with H437Y and H437R *rpoB* mutations being most frequently effective. Moreover, the *rpoB* mutations markedly activated (up to 70-fold at the transcriptional level) the cryptic/silent secondary metabolite biosynthetic gene clusters of these actinomycetes (20). Our present study with *P. agaridevorans* provides a molecular basis for the observed increase in the

transcripts of *cit* in the *rpoB* R485H mutant. Taken together, these findings indicate that certain *rpoB* mutations increase the productivity of antibiotics or enzymes mainly, if not solely, by enhancing the affinity of mutant RNA polymerases to the relevant promoters (reviewed by Alifano et al. [65] and Ochi [22]).

Because little CITase is produced by the wild-type strain T-3040, *cit* should be weakly expressed in this strain. *P. agaridevorans* T-3040 produces CIs from starch very slowly, and the amount in culture of 6-α-glucosyl transferase, which produces dextran-like α-(1→6)-glucosyl chains from starch, was small but negligible (40). A comparison of the *cit* gene loci of *P. agaridevorans* T-3040 and *Paenibacillus* sp. 598K showed that the *6gt31a* gene, which encodes 6-α-glucosyl transferase, is located immediately downstream from *cit* in the *Paenibacillus* sp. 598K genome and that these two genes are coexpressed by the same promoter (40). In contrast, the putative *6gt31a* gene in *P. agaridevorans* T-3040 is located distant from *cit* and on the opposite strand. Moreover, a member of the AraC family seems to regulate the transcription of the putative *6gt31a* gene, a regulator different from that for *cit*, suggesting that the CI production system of T3040 does not function well.

Cyclodextrin and cycloalternan are other types of bacterial cyclic oligosaccharides consisting of α-D-glucoses. *Klebsiella pneumoniae* has been reported to produce cyclodextrins extracellularly and intracellularly (66); these cyclodextrins are digested by a cyclodextrinase and metabolized by the maltose degradation pathway (66, 67). Cycloalternan-producing *Bacillus* sp. strain NRRL B-21195 has been reported to express an intracellular cycloalternan-degrading enzyme (68). CI-degrading enzymes must be dextranases, cleaving endo-type α-(1→6)-glucosidic bonds. In the CAZy database (39), dextranases were found in the GH66 and GH49 families. Because the draft genome of *P. agaridevorans* T-3040 contained no potential dextranase genes, except for *cit*, the lack of systems responsible for the production and digestion of CIs may have made CIs useless for strain T-3040, resulting in the degeneration of CITase-producing systems in this organism. This hypothesis may account for the silent or weak expression of the CITase gene in T-3040.

Sequencing of the genomes of *Streptomyces*, fungi, and myxobacteria has shown that each strain contains genes encoding the enzymes necessary to synthesize various potential secondary metabolites, but that these strains express only a fraction of these genes during fermentation (69, 70). The activation of silent or sleeping genes for the biosynthesis of bacterial secondary metabolites may therefore result in new methods applicable to industrial microbiology (18, 71, 72). Because the *rsmG* mutation activated the expression of the gene involved in CITase production, altering intracellular SAM levels by introducing an *rsmG* mutation may be feasible for activating dormant genes. Likewise, the introduction of *rpsL* and *rpoB* mutations could be used for a similar purpose. The discovery of a novel enzyme from a microbial source takes from months to years. Our method, involving activating the expression of silent or weakly expressed genes encoding dormant enzymes, does not require induced mutagenesis or gene-engineering techniques. Thus, the simplicity, wide applicability, and scalability of this method should facilitate not only industrial enzyme production but also the identification of novel enzymes from microbial sources.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Paenibacillus agaridevorans* T-3040 (assigned on the basis of 16S rRNA sequencing data [DDBJ accession no. LC042199] [73]), with 99.9% identity to that of the type strain, and formerly called *Bacillus circulans* T-3040 (FERMB-4132 [NBRC]), was obtained from Tetsuya Oguma (Kikkoman Co). Strain G22-10, a high producer of CITase, was derived from T-3040 by treating the spores with NTG (33). Spontaneous streptomycin-resistant and rifampin-resistant mutants were isolated by spreading the spores onto modified nutrient broth glucose (NG) agar plates (10 g nutrient broth, 10 g glucose, 5 mg $CuSO_4 \cdot 5H_2O$, 7.5 mg $FeSO_4 \cdot 7H_2O$, 3.6 mg $MnSO_4 \cdot 5H_2O$, 50 mg $MgSO_4 \cdot 7H_2O$, 15 mg $CaCl_2 \cdot 2H_2O$, and 9 mg $ZnSO_4 \cdot 7H_2O$ per liter) (74) containing various concentrations of the respective drugs. Similarly, spontaneous scandium-resistant mutants were isolated by plating onto modified NG agar plates containing 300 to 400 μg/ml of $ScCl_3 \cdot 6H_2O$ (purity, 99.9%). The growth of the parental strain was entirely inhibited by 300 μg/ml of $ScCl_3 \cdot 6H_2O$. All mutant strains were stored in 20% (vol/vol) glycerol at −80°C and routinely cultured on NG agar medium at 30°C for 3 to 5 days. LB-Dex medium

(Luria-Bertani [LB] medium containing 20 g dextran 40 [GE Healthcare] per liter) (33) was used for the production of CITase.

Determination of MICs. To determine MICs, full growth cultures were dotted on NG plates containing various concentrations of a drug and incubated at 30°C for 72 h. The minimum drug concentration able to fully inhibit growth was defined as the MIC.

Mutation analysis of the *rsmG*, *rpsL*, and *rpoB* genes. The *rsmG*, *rpsL*, and *rpoB* genes were each amplified by PCR, using the genomic DNA of each mutant strain as a template, synthetic oligonucleotide primers designed on the basis of sequences in several *Bacillus* strains (see Table S1 in the supplemental material), and Ex *Taq* (TaKaRa). Purified PCR products were directly sequenced with BigDye Terminator cycle sequencing kits (Perkin-Elmer Applied Biosystems, Foster City, CA).

Isolation of drug-resistant mutant strains from *Streptomyces coelicolor* A3(2). The *rsmG* mutant (KO-179) and *rsmG rpsL* double mutant (KO-945) were obtained as spontaneous drug-resistant mutant strains, as described previously (32). The triple (*rsmG rpsL rpoB*) mutant KO-1252 was derived from the double mutant KO-945 as a spontaneous rifampin-resistant colony, which developed by growing for 10 days on a glucose-yeast extract-malt extract (GYM) plate (75) containing 100 μg/ml of rifampin. Strain KO-1252 contained a mutation (1279G→A [Asp427→Asn]) in the *rpoB* gene, as determined by DNA sequencing.

Assay of CITase activity. CITase activity in the medium was determined as described previously (33). One unit of CITase activity was defined as the amount of enzyme that produced 1 nmol of the sum of CI-7, CI-8, and CI-9 per minute. The amount of CI (CI-7 to CI-12) in the culture supernatants was measured by high-pressure liquid chromatography (HPLC), as described previously (33). All CITase assays were repeated at least three times. The concentrations of protein (CITase) secreted into the medium were estimated using Bio-Rad Bradford protein assay kits (Bio-Rad, Hercules, CA), with bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as the standard.

Assay of amylase, protease, and agarase activities. The activities of amylase, protease, and agarase in the medium were determined as described previously (76, 77).

(i) Amylase. Culture supernatants (50 μl) obtained after centrifugation were mixed with 0.1 ml of soluble starch solution (50 mM Tris-HCl [pH 6.8], 0.5% soluble starch) and incubated at 30°C. A 4-μl aliquot of each reaction mixture was added to 0.1 ml of Lugol solution, and its absorbance at 700 nm (A_{700}) was measured. Units of amylase activity were expressed as $(A_{\text{blank}} - A_{\text{sample}}) \times A_{\text{blank}}{}^{-1} \times t^{-1} \times 100$, where t indicates the time (min) of the reaction and A_{blank} and A_{sample} indicate the absorbances at 700 nm of the blank (water) and the sample, respectively.

(ii) Protease. Culture supernatants (20 μl) obtained after centrifugation were mixed with reaction buffer (1.25% azocasein, 2 mM $CaC1_2$, 50 mM Tris-HCl [pH 7.5]) and incubated at 30°C for 20 min. The reactions were stopped by adding 0.2 ml of 10% trichloroacetic acid. The mixtures were centrifuged, and the absorbance of each supernatant was measured at 365 nm (A_{365}). Units of protease activity were expressed as $(A_{\text{sample}} - A_{\text{blank}}) \times t^{-1} \times 100$, where t indicates the time of the reaction and A_{blank} and A_{sample} indicate the absorbances at 365 nm of the blank (water) and the sample, respectively.

(iii) Agarase. Culture supernatants (40 μl) obtained after centrifugation were mixed with 360 μl of 50 mM sodium phosphate buffer (pH 7.0) containing 0.2% agarose (Agarose S; Nippon Gene Co., Ltd.). After incubating at 37°C for 30 to 60 min, the sample (100 μl) was mixed with 100 μl of dinitrosalicylic acid solution (650 mg dinitrosalicylic acid, 32.5 ml 2 M NaOH, 4.5 ml glycerol in 100 ml ultrapure water) and heated in boiling water for 10 min until the color developed. The reaction was stopped by cooling the sample on ice. Absorbance was measured at 540 nm (A_{540}). One unit of enzyme activity was defined as the amount of activity producing an A_{540} of 0.001 per minute and per ml enzyme solution.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and zymography. SDS-PAGE and enzyme zymography were performed as described previously (33). For SDS-PAGE, proteins were separated on 8% polyacrylamide gels and stained with Coomassie brilliant blue R-250 (CBB). For zymography, proteins were separated on 8% SDS-PAGE gels containing 1% blue dextran 2000 (GE Healthcare). The gels were subsequently washed twice with water to renature the proteins and soaked in 50 mM sodium acetate buffer (pH 5.5) at 37°C until active CITase was detected by the formation of a clear band on a blue background.

Total RNA preparation. Cells were treated with 1 ml chilled killing buffer (20 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 20 mM NaN_3), collected by centrifugation, and kept at −80°C. Total cellular RNA was prepared using Isogen reagent (Nippon Gene). Each frozen cell pellet was resuspended in 1 ml of Isogen reagent and incubated at 50°C for 10 min. After cooling, 0.2 ml of chloroform was added, and the sample was vortex and centrifuged at 16,000 × *g* for 10 min. A 0.6-ml aliquot of each aqueous phase (top layer) was transferred to a clean tube, 0.4 ml of chloroform was added, and the sample was mixed by repeated inversion and centrifuged at 16,000 × *g* for 10 min. A 0.6-ml aliquot of the aqueous phase (top layer) was transferred to a clean tube. The RNA in each tube was precipitated with an equal volume of isopropanol, rinsed with 70% ethanol, and suspended in an appropriate volume of diethyl pyrocarbonate (DEPC)-treated water.

Real-time quantitative PCR. Total RNA was prepared as described. Contaminating DNA was removed by incubating each total RNA sample (1 mg) with 1 U of DNase I (Invitrogen) at 25°C for 15 min. DNase I was inactivated by adding EDTA and heating to 65°C for 10 min. The RNA was reverse-transcribed using high-capacity RNA-to-cDNA kit reverse transcriptase (Applied Biosystems) and random hexamers according to the manufacturer's instructions. After terminating the reaction, the samples were diluted with an appropriate volume of water and analyzed using the 7300 real-time PCR system and Power SYBR green PCR master mix (Applied Biosystems). The synthetic oligonucleotide primers used to

amplify the CITase *S*-adenosylmethionine (SAM) synthetase gene (*metK*) and 16S rRNA (internal control) genes are listed in Table S1.

Primer extension. Total RNAs were prepared and the contaminating DNA was removed as described above. After ethanol precipitation, 50 μg RNA was incubated at 80°C for 15 min with 1 pmol of an infrared dye-labeled primer for the CITase gene (*cit*), IRD800-CITase (5′-IRD800-GGAGGGGATACGACGG ATGCGACG-3′) (Aloka). After annealing, 5 μl of 0.1 M dithiothreitol, 10 μl of 5-fold first-strand buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, and 15 mM $MgCl_2$), and 5 μl of deoxynucleoside triphosphate (dNTP) mix (10 mM [each] dATP, dGTP, dCTP, and dTTP) were added. To 50 μl of this reaction mixture, 200 U of SuperScript II (Invitrogen) was added and the mixture was incubated at 42°C for 1 h. Subsequently, 1 μl of RNase mixture (Ambion) was added, and the reaction mixture was further incubated at 37°C for 30 min. After ethanol precipitation, primer extension and sequencing reactions with the corresponding primers were run on polyacrylamide gels, followed by analysis using the DNA sequencing system LIC-4200L(S)-2 (LI-COR).

***In vitro* protein synthesis.** Cells grown to late exponential phase (A_{600} of 0.4) in LB-Dex medium were collected by centrifugation and washed with standard buffer (10 mM Tris-HCl [pH 7.7] containing 2 mM phenylmethylsulfonyl fluoride, 10 mM magnesium acetate, 30 mM ammonium acetate, and 6 mM 2-mercaptoethanol). Washed ribosomes were prepared (41), followed by the cell-free synthesis of chloramphenicol acetyltransferase (CAT), as described previously (78).

Determination of SAM level. The levels of intracellular SAM in *P. agaridevorans* wild-type and mutant strains were determined by reverse-phase HPLC, as described previously (34, 79). Cells grown to various growth phases were harvested, transferred to petri dishes containing 10 ml of 1 M formic acid, and allowed to stand at 4°C for 1 h. The formic acid was collected, filtered through a membrane filter (pore size, 0.45 μm), and lyophilized. Each lyophilized sample was dissolved in a small volume of water and analyzed on a TSKgel ODS-100V column (4.6 mm by 250 mm; Tosoh, Tokyo, Japan). To normalize the number of picomoles of SAM per milliliter of culture to the number of picomoles per number of cells, intracellular SAM levels were expressed as pmol/optical density (OD) unit, where 1 OD unit was defined as the number of cells that would produce an absorbance of 1 at 600 nm (A_{600} of 1.0) if suspended in 1 ml.

Metabolome analysis. Cells grown at 30°C for 12 h or 48 h in LB-Dex medium were harvested by filtration using membrane filters (pore size, 0.45 μm; Millipore), washed twice with deionized water, immediately transferred to a petri dish containing 10 ml of 1 M formic acid, and allowed to stand at 4°C for 1 h. The formic acid was collected, filtered through a membrane filter, and lyophilized. The lyophilized samples were dissolved in a small amount of deionized water for metabolome analysis. Metabolome analysis was performed by a facility service at Human Metabolome Technology Inc. (Tsuruoka, Japan). Capillary electrophoresis-time of flight mass spectrometry (CE-TOFMS) was performed using an Agilent CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany), equipped with an Agilent 6210 time of flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-electrospray ionization (ESI)-MS sprayer kit. The systems were controlled by Agilent G2201AA ChemStation software version B.03.01 for CE. The metabolites were analyzed using a fused silica capillary column (50-μm inner diameter by 80-cm total length), with commercial electrophoresis buffers (solution identifiers H3301-1001 for cation analysis and H3302-1021 for anion analysis; Human Metabolome Technologies) as the electrolyte. Samples were injected at a pressure of 5 kPa for 10 s (approximately 10 nl) for cation analysis and for 25 s (approximately 25 nl) for anion analysis. The spectrometer was scanned from *m/z* 50 to 1,000. Other conditions were as described previously (80, 81). Peaks were extracted using MasterHands automatic integration software (Keio University, Tsuruoka, Japan) to obtain peak information, including *m/z*, migration time for CE-TOFMS measurement (MT), and peak area (82). Signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites were excluded, and the remaining peaks were annotated with putative metabolites from the HMT metabolite database, based on their MTs and *m/z* values determined by TOFMS. The tolerance range for the peak annotation was configured at $\pm$0.5 min for MT and $\pm$10 ppm for *m/z*. Peak areas were normalized against those of the internal standards, and the resultant relative areas were further normalized by sample amount. Hierarchical cluster analysis (HCA) and principal-component analysis (PCA) were performed using PeakStat and SampleStat proprietary software, respectively. To normalize the number of picomoles of metabolites per milliliter of culture to the number of picomoles per number of cells, intracellular metabolite levels were expressed as pmol/optical density (OD) unit, where 1 OD unit was defined as the number of cells that would produce an A_{600} of 1 if suspended in 1 ml.

Draft genome analysis. A DNA library with a median insert size of 500 bp was constructed in a multiplexed paired-end read format, according to the Illumina protocols. Whole-genome sequencing, generating 100-bp paired-end reads, was performed using an Illumina Genome Analyzer IIx according to the manufacturer's protocol. *De novo* assembly was performed using Velvet (83) with parameters optimized by the VelvetOptimiser (https://github.com/tseemann/VelvetOptimiser). Coding sequences were predicted by MiGAP (http://www.migap.org/). Gene functions were annotated by a blastp search against an in-house protein database consisting of all predicted proteins in RefSeq representative genomes. Carbohydrate-related enzymes were also annotated using the CAZy database (http://www.cazy.org/) (39) and the dbCAN annotation server (http://csbl.bmb.uga.edu/dbCAN/) (84).

Large-scale production of CITase. Fermentation was scaled up by C-I-Bio Ltd. (Okinawa), using a 90-liter jar fermentor (medium, 60 liters; MSJ-U2W type; B. E. Marubishi Co., Kasugai, Japan) and a 300-liter tank (medium 200 L; Tsukayama Stainless Co., Okinawa, Japan).

Reproducibility. All experiments were performed at least three times to confirm reproducibility, except for the experiments in Data Set S1, which were performed only once.

Accession number(s). The draft genome sequence of *Paenibacillus agaridevorans* T-3040 has been deposited in the DDBJ/EMBL/GenBank databases under accession numbers BDQX01000001 to BDQX01000467.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00188-18.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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K.O. designed the project, analyzed the data, and wrote the paper; K.F. and Y.T. isolated drug-resistant mutants and analyzed CITase activity. T.H. and T.I. performed biochemical and transcriptional analyses of protein synthesis and *cit*, respectively. Y.H. assayed intracellular SAM level, and K.M. and K.K. studied the cultivation conditions for CITase production. Y.S., T.M., N.F., and H.Y. conducted genome analysis of *P. agaridevorans*. S.G. conducted the scale-up experiments.

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