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The *Bacillus subtilis tyrZ* Gene Encodes a Highly Selective Tyrosyl-tRNA Synthetase and Is Regulated by a MarR Regulator and T Box Riboswitch

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ABSTRACT

Misincorporation of D-tyrosine (D-Tyr) into cellular proteins due to mischarging of $tRNA^{Tyr}$ with D-Tyr by tyrosyl-tRNA synthetase inhibits growth and biofilm formation of *Bacillus subtilis*. Furthermore, many *B. subtilis* strains lack a functional gene encoding D-aminoacyl-tRNA deacylase, which prevents misincorporation of D-Tyr in most organisms. *B. subtilis* has two genes that encode tyrosyl-tRNA synthetase: *tyrS* is expressed under normal growth conditions, and *tyrZ* is known to be expressed only when *tyrS* is inactivated by mutation. We hypothesized that *tyrZ* encodes an alternate tyrosyl-tRNA synthetase, expression of which allows the cell to grow when D-Tyr is present. We show that TyrZ is more selective for L-Tyr over D-Tyr than is TyrS; however, TyrZ is less efficient overall. We also show that expression of *tyrZ* is required for growth and biofilm formation in the presence of D-Tyr. Both *tyrS* and *tyrZ* are preceded by a T box riboswitch, but *tyrZ* is found in an operon with *ywaE*, which is predicted to encode a MarR family transcriptional regulator. Expression of *tyrZ* is repressed by YwaE and also is regulated at the level of transcription attenuation by the T box riboswitch. We conclude that expression of *tyrZ* may allow growth when excess D-Tyr is present.

IMPORTANCE

Accurate protein synthesis requires correct aminoacylation of each tRNA with the cognate amino acid and discrimination against related compounds. *Bacillus subtilis* produces D-Tyr, an analog of L-Tyr that is toxic when incorporated into protein, during stationary phase. Most organisms utilize a D-aminoacyl-tRNA deacylase to prevent misincorporation of D-Tyr. This work demonstrates that the increased selectivity of the TyrZ form of tyrosyl-tRNA synthetase may provide a mechanism by which *B. subtilis* prevents misincorporation of D-Tyr in the absence of a functional D-aminoacyl-tRNA deacylase gene.

Bacteria must appropriately aminoacylate each tRNA with the correct amino acid in order to maintain accurate translation (1). It was shown previously that growth of *Bacillus subtilis* is inhibited in the presence of D-tyrosine (D-Tyr) as a result of mischarging of $tRNA^{Tyr}$ with D-Tyr by tyrosyl-tRNA synthetase and subsequent incorporation into proteins (2). Misincorporation of D-Tyr into proteins also inhibits biofilm formation, as a result of growth inhibition, in undomesticated strains of *B. subtilis* (3; see also the accompanying paper by Leiman et al. [4]). D-Tyr competitively inhibits prephenate dehydrogenase, which is involved in biosynthesis of L-tyrosine (L-Tyr) (5). Therefore, the cellular concentration of L-Tyr decreases in the presence of D-Tyr, which increases the probability that $tRNA^{Tyr}$ will be mischarged with D-Tyr by tyrosyl-tRNA synthetase (6).

D-Tyr is produced by *B. subtilis* during stationary phase, which influences peptidoglycan biosynthesis (7). Furthermore, *Bacillus* species produce antibiotic peptides such as iturin and bacillomycin that contain D-Tyr (8). Many organisms prevent misincorporation of D-Tyr during protein synthesis by expressing the *dtd* gene, which encodes D-aminoacyl-tRNA deacylase, an enzyme that removes D-Tyr from $tRNA^{Tyr}$ (2, 9, 10). However, many laboratory strains of *B. subtilis* contain mutations in the *dtd* gene (3).

B. subtilis has two tyrosyl-tRNA synthetase-encoding genes: *tyrS* is expressed during vegetative growth, and *tyrZ* is known to be expressed only when *tyrS* is inactivated by mutation (11, 12). We hypothesized that TyrZ may allow the cell to maintain accurate translation when D-Tyr is present. The *tyrZ* gene is located downstream of the *ywaE* gene, which is predicted to encode a MarR family transcriptional regulator (13), and *tyrZ* is predicted to be cotranscribed with *ywaE* (Fig. 1).

Members of the MarR family of transcriptional regulators regulate many different genes involved in metabolic pathways, stress responses, virulence, and transport of harmful compounds (13–17). These regulators typically repress transcription initiation by binding to a palindromic (or pseudopalindromic) sequence that overlaps the promoter. DNA binding by the transcriptional regulator is often inhibited by binding of an anionic phenolic com-

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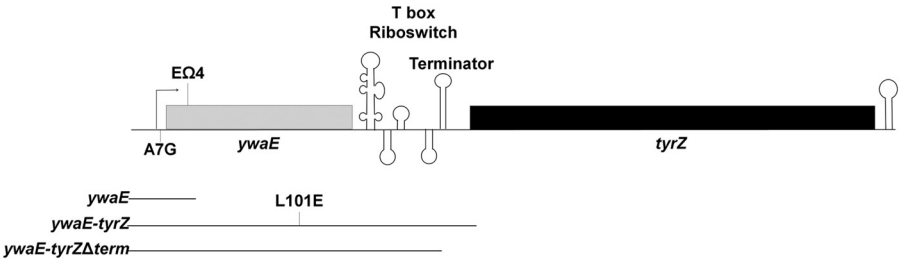

FIG 1 *ywaE-tyrZ* operon organization and mutations. The *ywaE* gene is depicted by a gray box, and the *tyrZ* gene is depicted by a black box. The T box riboswitch is depicted by a series of stem-loops between the *ywaE* and *tyrZ* genes, with the intrinsic transcriptional terminator labeled. The promoter is depicted by an arrow, and a terminator helix for the operon is depicted by a stem-loop. The locations of the spontaneous mutations in the *ywaE* gene used in the study (EΩ4 and E^{opA7G}) are indicated. The DNAs included in the *lacZ* reporter fusions are indicated below the operon. The location of the L101E mutation is indicated in the fusion for the *ywaE*L101E-*tyrZ-lacZ* fusion. All fusions were constructed in the absence (wild type) or presence of the A7G mutation.

pound to the protein (18–20). The predicted cotranscription of *ywaE* and *tyrZ* raised the possibility that YwaE could regulate *tyrZ* transcription initiation.

Both *tyrS* and *tyrZ* are preceded by a T box riboswitch (12), which is located in the leader region of the monocistronic *tyrS* gene and between *ywaE* and *tyrZ*, respectively. Expression of genes regulated by a T box riboswitch is dependent on direct interaction between the riboswitch and the cognate uncharged tRNA, which determines whether the RNA folds into the helix of an intrinsic transcriptional terminator or a competing antiterminator (21). A triplet sequence in the riboswitch, termed the specifier sequence, which corresponds to the codon for the amino acid related to the downstream gene, pairs with the anticodon of either the charged or uncharged forms of the cognate tRNA (21–23). The free acceptor end of the uncharged tRNA binds to and stabilizes the antiterminator structure, which prevents terminator formation (22). Charged tRNA is unable to stabilize the antiterminator, which allows formation of the more stable intrinsic transcriptional terminator, resulting in premature termination of transcription (22).

The goal of the present study was to investigate the physiological role of the *B. subtilis tyrZ* gene. We hypothesized that TyrS and TyrZ might have different kinetic properties with respect to incorporation of D-Tyr and L-Tyr. We tested whether the ability to express *tyrZ* affects inhibition of growth and biofilm formation by D-Tyr. In addition, genetic analysis of the *ywaE-tyrZ* operon was conducted to investigate the regulation of *tyrZ* expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in the study are listed in Table S1 in the supplemental material. *B. subtilis* strains were grown in 2× YT broth (24), tryptose blood agar base (TBAB; Difco), or Spizizen minimal medium with glucose as a carbon source (25). Biofilm-forming strains of *B. subtilis* were grown in modified MSgg minimal medium with glycerol as a carbon source (3, 26). *Escherichia coli* strains used for plasmid propagation and protein purification were grown in LB medium (24). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 30 μg/ml; neomycin, 5 μg/ml; erythromycin, 1 μg/ml; and chloramphenicol, 5 μg/ml for selection or 0.1 μg/ml for induction. Required amino acids were supplied at 50 μg/ml. Growth was carried out at 37°C, unless otherwise stated.

Genetic techniques. PCRs were carried out using *Taq* DNA polymerase (Invitrogen). Chromosomal DNA from strain BR151 or mutant derivatives was used as the template for PCR, unless otherwise stated. All restriction enzymes and T4 DNA ligase were supplied by New England BioLabs and used according to the manufacturer's recommendations. Plasmid DNA was purified using Wizard columns (Promega).

All *lacZ* reporter fusions were constructed using plasmid pFG328 (21) and PCR to generate insert DNA fragments. Oligonucleotide primers (Integrated DNA Technologies) used for PCR are listed in Table S2 in the supplemental material. Constructs were inserted into the BamHI and XbaI sites of pFG328 and verified by DNA sequencing (Genewiz). The *ywaE*L101E-*tyrZ-lacZ* fusion was generated by site-directed mutagenesis using plasmid pFG328-*ywaE-tyrZ* as the template. Plasmids were introduced into XL2-Blue ultracompetent cells (Stratagene) by transformation per the manufacturer's recommendations, with selection for ampicillin resistance.

B. subtilis strains were complemented with a functional *dtd* gene in which the mutant AAG lysine codon was replaced with the AUG start codon. Upstream and downstream fragments, generated by PCR, were used as the PCR template of the final *dtd* gene product (see Table S2 in the supplemental material), which was inserted into the BamHI and XbaI sites of plasmid pFG328 to generate plasmid pFG328-*dtd*.

All *lacZ* reporter gene fusions and plasmid pFG328-*dtd* were introduced into the SPβ genome by transformation of strain ZB370A (27). Transducing phage was purified by passage through strain ZB449 and introduced into the appropriate background strain by transduction, with selection for chloramphenicol or erythromycin resistance.

The coding regions of *B. subtilis tyrS* and *tyrZ* were amplified by PCR (see Table S2 in the supplemental material) and inserted into the NcoI and XhoI sites of the expression vector pET33b+ (Novagen), which adds a C-terminal His_6 tag to each enzyme for purification. Plasmids were inserted into BL21(DE3) cells by transformation with selection for kanamycin resistance.

Overexpression and purification of TyrS and TyrZ. BL21(DE3) cells harboring the appropriate plasmid were grown overnight statically at 37°C and diluted 170-fold with fresh LB medium containing the appropriate antibiotic. Cells were grown at 37°C with agitation until the A_{600} reached ~0.6. Isopropyl-β-D-thiogalactopyranoside (1 mM) was added, and cells were harvested after 3 h of growth at 37°C. His_6-tagged proteins were purified according to the Qiagen QIAexpressionist high-stringency protocol. Purification of TyrZ required 600 mM NaCl in the lysis and wash buffers. Purified proteins were dialyzed and stored in 50 mM Tris-HCl (pH 7.5), 140 mM KCl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 0.2 μg/ml bovine serum albumin (BSA). Protein concentration was determined using the Bio-Rad protein assay and was 840 μg/ml for TyrS and 1.2 mg/ml for TyrZ. Denaturing protein gel analysis revealed that the proteins exhibited the molecular weight expected of monomers and that purity was >80%.

Active site titration assay. The concentration of active TyrS and TyrZ was determined by incubating TyrS (4.2 μg) and TyrZ (6.0 μg) at 37°C for 10 min with 50 μM [^{14}C]tyrosine (482 mCi/mmol; PerkinElmer) in the presence of 100 mM HEPES (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, and 1.1 μl pyrophosphatase (200 U/ml; Roche). The reaction mixture was passed through a nylon filter (Whatman Protran BA85; 0.45-μm

TABLE 1 Kinetic analysis of TyrS and TyrZ

Enzyme	L-Tyr			D-Tyr			Specificity[a]
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	
TyrS	33 ± 4	21 ± 1	$6.4 \times 10^{-1} \pm 5 \times 10^{-2}$	89 ± 3	2.3 ± 0.3	$2.9 \times 10^{-2} \pm 1 \times 10^{-2}$	22
TyrZ	36 ± 4[b]	6.7 ± 0.3[b]	$8.7 \times 10^{-2} \pm 3 \times 10^{-3}$	>300	>0.7	$1.7 \times 10^{-4} \pm 3 \times 10^{-5}$[c]	510

[a] Specificity was determined as follows: (k_{cat}/K_m) L-Tyr/(k_{cat}/K_m) D-Tyr.
[b] Values were extrapolated ignoring substrate inhibition at concentrations of L-Tyr of >0.3 mM.
[c] Value extrapolated in the absence of saturating concentrations of D-Tyr due to solubility limits of D-Tyr.

pore size, 25-mm diameter) under vacuum and then washed with 3 ml wash buffer (50 mM HEPES [pH 7.2], 15 mM KCl, 5 mM $MgCl_2$). The filter was dried at 85°C followed by liquid scintillation counting. The active concentrations of TyrS and TyrZ were 3.7 μM and 8.0 μM, respectively.

ATP-$^{32}PP_i$ exchange assay. TyrS (50 nM) and TyrZ (75 to 100 nM) were incubated with the amino acid indicated in 100 mM HEPES (pH 7.5), 140 mM KCl, 10 mM $MgCl_2$, 2 mM NaF, 10 mM dithiothreitol (DTT), 2 mM ATP, and 2 mM $^{32}PP_i$ (3 to 5 cpm/pmol; PerkinElmer) in a total volume of 110 μl. The concentration of L-Tyr ranged from 0.3 to 250 μM for TyrS and 1 to 3,000 μM for TyrZ, and D-Tyr ranged from 1 to 2,000 μM for TyrS and 300 to 3,500 μM for TyrZ. At 0.5- to 1-min intervals, samples (25 μl) of the reaction mixture were removed and added to 970 μl of a quenching solution (1% charcoal, 5.6% perchloric acid, 75 mM $NaPP_i$). The reaction mixture was filtered through 3-mm Whatman cellulose chromatography filter paper discs (2.4-cm diameter) under vacuum and then washed with 15 ml of water and 5 ml of 95% ethanol. The filters were dried at 85°C followed by liquid scintillation counting. Kinetic parameters were determined by fitting the data to Michaelis-Menten kinetics by nonlinear regression (Kaleidagraph; Synergy Software) and reported as averages ± standard errors of three independent repeats.

Insertional inactivation of *tyrS* and *tyrZ*. Plasmid pBLG1Neo, a derivative of plasmid pMMN13 (28), contains a fragment of *tyrS* extending from 327 to 984 bp downstream of the AUG start codon. The neomycin resistance cassette from pBEST501 (29) was inserted at the BclI site 617 bp downstream of the AUG in *tyrS*. The plasmid was introduced into strain BR151 by transformation. Transformants were selected for neomycin resistance and screened for chloramphenicol sensitivity to generate BR151-SKO strains. A similar strategy was used to construct plasmid *tyrZ*-pMMNneo to generate strain BR151-ZKO (30). Strains NCIB 3610-ZKO and NCIB 3610-SKO were constructed by SPP1 phage transduction and BR151-ZKO or BR151-SKO-EΩ4 as donor strain, respectively, with selection for neomycin resistance (31). To restore the *tyrS* gene and generate strains BR151-E^{opA7G} and BR151-EΩ4, chromosomal DNA from strain TyrSupX, in which the *cat* resistance cassette is inserted into the *acsA* gene upstream of *tyrS* (32), was introduced into strains BR151-SKO-E^{opA7G} and BR151-SKO-EΩ4 by transformation. Transformants were selected for chloramphenicol resistance and screened for neomycin sensitivity. Strain 3610-E^{V164E} was constructed by SPP1 transduction using strain TyrSupX (32) as the donor strain and 3610-SKO as the recipient strain with selection for chloramphenicol resistance. Expression of *tyrZ* was confirmed by reverse transcription-PCR (RT-PCR) (see Fig. S1 in the supplemental material).

β-Galactosidase assays. *B. subtilis* cells harboring *lacZ* fusions were grown until early exponential phase in Spizizen minimal medium (25). Cells were harvested and resuspended in fresh medium in the presence or absence of 2 μg/ml 4-amino-L-phenylalanine (4-ALP), to induce tyrosine starvation. Samples were collected after 3 h of incubation, permeabilized by toluene, and assayed as described by Miller (24). Growth and β-galactosidase assays were repeated at least 3 times.

Zone-of-growth-inhibition assays. Strains were grown in Spizizen minimal medium to exponential phase and then diluted 30-fold. A lawn of bacterial growth was generated by combining 0.1 ml of diluted culture with 3 ml of minimal medium solidified with 1% Bacto agar (Difco), and the mixture was poured onto the surface of a minimal medium plate. Filter discs (1.2 cm) were placed onto the surface, and D-Tyr (0, 2.5, 5, 12.5, and 25 μg) was applied to each disc. Bacterial growth near the filter disc was monitored after 24 and 48 h. Assays were repeated at least 3 times.

Biofilm formation assay. Strains were grown at 37°C with agitation overnight and then diluted 1,000-fold in modified MSgg broth or spotted (5 μl) onto modified MSgg plates solidified with 1.5% Bacto agar in the presence or absence of 6 μM D-Tyr. The cultures were grown at 25°C without agitation for 1 to 5 days. Biofilms were photographed after 3 days at ×10 magnification using a dissecting microscope (Cambridge Instruments model no. Z30L) and a Samsung Galaxy S5 camera.

RESULTS

TyrZ is more selective than TyrS for L-Tyr. The presence of two genes, *tyrS* and *tyrZ*, that encode tyrosyl-tRNA synthetase led us to determine the kinetic properties of each enzyme. The ability of *B. subtilis* TyrS and TyrZ to activate L-Tyr or D-Tyr was assayed by ATP-PP_i exchange (Table 1). This assay measures the first step of aminoacylation in which the amino acid and ATP condense to form the enzyme-bound aminoacyl-adenylate. This step is reversible, and incubation in the presence of radiolabeled pyrophosphate permits the rate of formation of radiolabeled ATP to serve as a readout of activation of L- or D-Tyr. TyrS activated both L- and D-Tyr. However, activation of D-Tyr showed a 2.7-fold increase in the K_m and a 9-fold decrease in the k_{cat} compared to the activation of L-Tyr. Comparison of the respective k_{cat}/K_m values revealed that TyrS is 22-fold more specific for the activation of L-Tyr over D-Tyr. In contrast, TyrZ activated L-Tyr but was inhibited by concentrations of L-Tyr of >0.3 mM. The individual K_m and k_{cat} values could not be determined for D-Tyr activation by TyrZ due to the limit of solubility of D-Tyr, but extrapolation to determine k_{cat}/K_m indicated that TyrZ is 510-fold more specific for the activation of L-Tyr than D-Tyr. Comparison of the k_{cat}/K_m for each enzyme showed that TyrZ is 7-fold less efficient than TyrS at activating L-Tyr, whereas TyrZ is 170-fold less efficient than TyrS during the activation of D-Tyr. These results indicate that TyrZ has higher selectivity than TyrS for L-Tyr over D-Tyr but has a lower overall catalytic efficiency.

Compensatory mutations occur in *ywaE* when *tyrS* is inactivated. The different selectivity of TyrS and TyrZ led us to determine how expression of *tyrS* and *tyrZ* affected growth in the presence of D-Tyr. Each gene was inactivated by insertion of a neomycin resistance cassette, followed by selection for neomycin resistance in the absence of D-Tyr. Disruption of either gene did not significantly affect the growth rate in rich or minimal medium (data not shown). Mutants in which *tyrS* and *tyrZ* were inactivated simultaneously could not be recovered (T. M. Henkin and F. J. Grundy, unpublished results). When *tyrZ* was inactivated in strain BR151, colonies were isolated after 24 h of growth, yielding strains

TABLE 2 Expression of *ywaE-lacZ* and *ywaE*^{opA7G}*-lacZ* fusions

Strain	*ywaE*[b]	β-Galactosidase activity[a]		Fold change[c]
		ywaE-lacZ	*ywaE*^{opA7G}*-lacZ*	
BR151	+	0.73 ± 0.15	92 ± 16	130
ZKO	+	0.83 ± 0.23	71 ± 3.2	86
SKO-E^{opA7G}	+	1.0 ± 0.20	90 ± 19	90
SKO-EΩ4	−	37 ± 6.2	310 ± 43	8.4
EΩ4	−	82 ± 5.2	260 ± 9.3	3.2

[a] Fusions were integrated in single copy into *B. subtilis* strains. Cells were grown in minimal medium until late exponential phase and then assayed for β-galactosidase activity (Miller units). The values reported are averages of three repeats ± standard errors.
[b] Indicates if the strain has a functional *ywaE* gene.
[c] Fold change is the ratio of β-galactosidase activity for *ywaE*^{opA7G}*-lacZ* to *ywaE-lacZ*.

designated ZKO. In contrast, after inactivation of *tyrS*, colonies appeared only after 48 to 72 h of incubation, yielding strains designated SKO. We hypothesized that a second mutation might have occurred in the *ywaE* gene based on its location upstream of *tyrZ* and the prediction that *ywaE* encodes a MarR regulator. Furthermore, introduction of a wild-type copy of the *ywaE* gene carried on an SPβ prophage resulted in loss of growth of SKO strains, presumably because of repression of *tyrZ* expression. We therefore sequenced the *ywaE* gene of multiple independent SKO isolates and identified nine independent mutations in the *ywaE* gene. Three different mutations were identified in the coding region, including a 4-nucleotide (nt) insertion 32 nt downstream of the start codon (designated EΩ4) predicted to cause a frameshift, G264A, which creates an in-frame stop codon, and G232A, which converts an alanine to threonine at a position that corresponds to the helix-turn-helix domain in other MarR homologs (13, 20). Five independent mutations were identified in the Shine-Dalgarno (SD) region of *ywaE* that are predicted to disrupt translation initiation. A separate A7G mutation was identified between the predicted −10 region of the promoter and the SD sequence (designated E^{opA7G}). Inactivation of *tyrS* in strain NCIB 3610 (3610) did not affect the growth rate in rich or minimal medium and resulted in a single *ywaE* T491A mutation (3610-SKO-E^{V164E}) that converts valine to glutamic acid at a position corresponding to the dimerization domain of other MarR homologs (13, 20). These mutations are predicted to either disrupt the function of YwaE or decrease expression of *ywaE*.

YwaE represses transcription of the *ywaE-tyrZ* operon. TyrS, which is expressed during vegetative growth (12), is more efficient than TyrZ at aminoacylation of tRNA^{Tyr} with L-Tyr. This suggested that expression of *tyrZ* might be regulated, to enable primary utilization of TyrS. The identification of mutations in *ywaE* in strains in which *tyrZ* was expressed suggested that YwaE represses transcription of the *ywaE-tyrZ* operon. Expression of a *ywaE-lacZ* fusion was low in strains that have a functional *ywaE* gene (BR151, ZKO, and SKO-E^{opA7G}) (Table 2). However, expression of the fusion increased 51-fold when the *tyrS* and *ywaE* genes were inactivated (SKO-EΩ4) and 110-fold when only the *ywaE* gene was inactivated (EΩ4), in comparison to expression of this fusion in BR151. These results are consistent with the prediction that YwaE represses transcription of the *ywaE-tyrZ* operon and are further supported by RT-PCR analysis of *tyrZ* transcript levels (see Fig. S1 in the supplemental material).

Identification of an operator sequence important for YwaE-mediated repression. Since YwaE represses transcription of *ywaE-tyrZ*, and MarR family proteins recognize palindromic operator sites in the promoter regions of their target genes, we compared the promoter regions of *ywaE-tyrZ* operons in organisms related to *B. subtilis* (Fig. 2A). A conserved, pseudopalindromic 13-nt sequence (TCATAGCTAATGA) was identified between the putative −10 element and SD sequence (Fig. 2B). A role for this sequence in YwaE-mediated repression was supported by identification of the A7G mutation within this sequence as an allele that

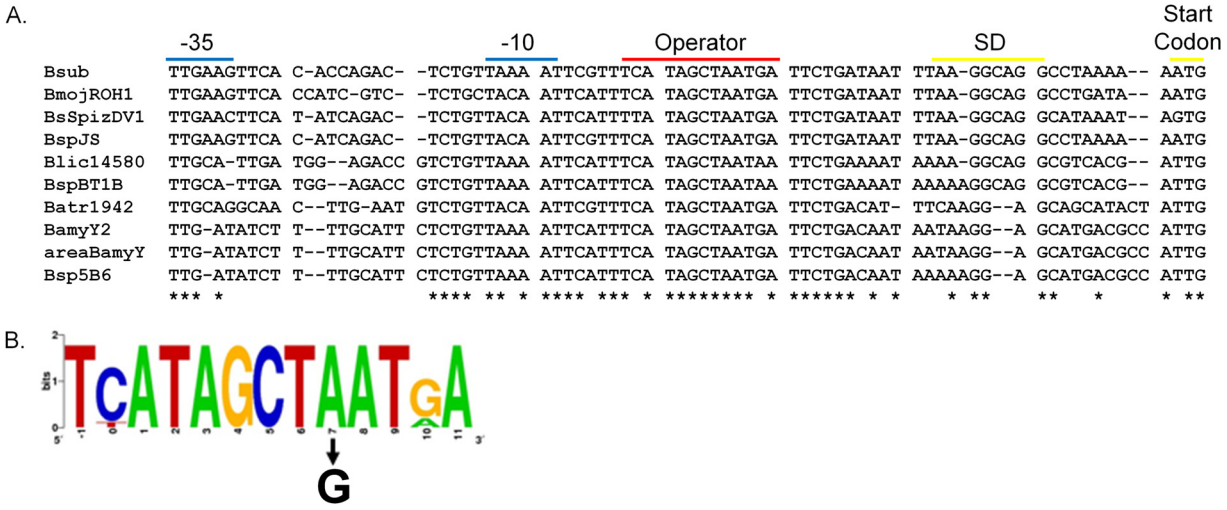

FIG 2 Identification of a putative operator sequence. The promoter regions of *ywaE* genes found upstream of *tyrZ* genes in organisms related to *B. subtilis* were aligned. (A) Sequences were identified using BLASTp and aligned using the ClustalW algorithm. The putative −35 and −10 sequences, the SD sequence and start codons, and the predicted operator sequence are indicated. Bsub, *Bacillus subtilis* subsp. *subtilis* strain 168; BmojROH1, *Bacillus mojavensis* ROH1; BsSpizDV1, *Bacillus subtilis* subsp. *spizizenii* DV1; BspJS, *Bacillus* sp. strain JS; Blic14580, *Bacillus licheniformis* DSM 13; BspBT1B, *Bacillus* sp. strain BT1B; Batr1942, *Bacillus atrophaeus* 1942; BamyY2, *Bacillus amyloliquefaciens* Y2; areaBamyY, *Bacillus amyloliquefaciens* subsp. *plantarum* YAU B9601-Y2; Bsp5B6, *Bacillus* sp. strain 5B6. (B) A WebLogo of the predicted operator sequence was created using the WebLogo sequence generator program (42, 43). An arrow depicts the location of the A7G mutation identified.

TABLE 3 Expression of *ywaE-tyrZ-lacZ* fusions

Fusion[a]	Strain	*ywaE*[b]	β-Galactosidase activity		Induction (fold)[c]
			− 4-ALP	+ 4-ALP	
tyrS-lacZ	BR151	+	24 ± 4	140 ± 20	5.8
ywaE-tyrZ-lacZ	BR151	+	0.18 ± 0.02	1.3 ± 0.39	7.2
	EΩ4	−	0.27 ± 0.01	4.2 ± 0.15	15
*ywaE*L101E-*tyrZ-lacZ*	BR151	+	0.17 ± 0.02	1.5 ± 0.31	8.5
	SKO-EΩ4[d]	−	11 ± 2.0	49 ± 5.5	4.4
	EΩ4	−	3.9 ± 1.3	55 ± 3.8	14
*ywaE-tyrZ*Δ*term-lacZ*	BR151	+	1.1 ± 0.17	1.2 ± 0.19	1.1
	EΩ4	−	1.8 ± 0.06	1.3 ± 0.22	0.72

[a] Cells harboring each fusion were grown in the presence or absence of 4-amino-L-phenylalanine (4-ALP) until late exponential phase and then assayed for β-galactosidase activity (Miller units). The values reported are averages of three repeats ± standard errors.
[b] Indicates if the strain has a functional *ywaE* gene.
[c] Induction by 4-ALP was determined by the ratio of β-galactosidase activity in the presence of 4-ALP to activity in the absence of 4-ALP.
[d] This strain expresses only *tyrZ*.

permitted inactivation of *tyrS* (presumably by promoting expression of *tyrZ*, which is required in the absence of *tyrS*).

We verified the importance of this sequence for repression by YwaE by monitoring expression of a *ywaE*^{opA7G}*-lacZ* fusion that included the A7G mutation (Table 2). Activity from this fusion increased at least 86-fold relative to expression of the wild-type fusion in strains that contain a functional *ywaE* gene. These results indicate that the operator sequence is important for repression by YwaE. Activity of this fusion was 3-fold higher in strains lacking a functional *ywaE* than in strain BR151, which suggests that the A7G mutation does not completely inactivate the operator sequence. Expression of the *ywaE*^{opA7G}*-lacZ* fusion was at least 3-fold higher than that of the *ywaE-lacZ* fusion in strains that have the EΩ4 mutation, indicating that this mutation does not completely inactivate *ywaE* or that another regulatory protein may interact with the operator sequence. The EΩ4 mutation results in a frameshift that causes a premature stop codon 61 nt downstream of the AUG. However, there is another start codon downstream of the EΩ4 mutation. If translation initiated at this codon (albeit with low efficiency because of the absence of an obvious SD sequence), the resulting protein would contain a 23-amino-acid truncation at the N terminus, which is not highly conserved in other MarR homologs.

The *tyrZ* T box riboswitch is functional. The *tyrZ* gene is preceded by a T box riboswitch element that is predicted to respond to the aminoacylation status of tRNA^{Tyr}, such that readthrough of an intrinsic transcriptional terminator is promoted by uncharged tRNA^{Tyr} (12, 21). We predicted that tRNA^{Tyr}-mediated regulation of *tyrZ* is superimposed upon repression of transcription by YwaE. 4-ALP is a tyrosine analog that inhibits biosynthesis of L-Tyr but does not get charged onto tRNA^{Tyr}, resulting in decreased aminoacylation of tRNA^{Tyr} (32). Expression of the *tyrS* gene increases in the presence of uncharged tRNA^{Tyr} (21), and a *tyrS-lacZ* fusion was induced 6-fold by 4-ALP (Table 3).

A *ywaE-tyrZ-lacZ* fusion that fused the *ywaE* gene and the *tyrZ* leader region to the *lacZ* gene was inserted into an SPβ prophage. Expression was induced 7- and 15-fold by 4-ALP in strains BR151 and EΩ4, respectively, but expression levels were very low. Introduction of this fusion, which contains the entire *ywaE* gene, into strains BR151, ZKO, and SKO-E^{opA7G} results in two functional copies of *ywaE* and in strain EΩ4 results in one functional copy. Repression of transcription by YwaE was relieved by construction of a *ywaE*L101E-*tyrZ-lacZ* fusion in which a conserved leucine in the putative DNA recognition domain was mutated to glutamic acid (Table 3). Since strain SKO-EΩ4 is dependent on *tyrZ* expression (and relief of repression by YwaE), we were unable to introduce the *ywaE-tyrZ-lacZ* fusion into this strain. However, the ability to introduce the *ywaE*L101E-*tyrZ-lacZ* fusion into strain SKO-EΩ4 indicated that the *ywaE*L101E allele disrupts YwaE function.

Regulation of the *ywaE*L101E-*tyrZ-lacZ* fusion in strains lacking *ywaE* is predicted to be dependent solely on the *tyrZ* T box riboswitch. Induction of expression of this fusion by 4-ALP was similar to that of the *ywaE-tyrZ-lacZ* fusion in all strains. The basal levels of activity increased 14-fold in strain EΩ4, which further confirms that YwaE encoded by the fusion is inactive. These data suggest that readthrough of the *tyrZ* T box terminator occurs under conditions that are predicted to increase the concentration of uncharged tRNA^{Tyr} in the cell. Expression of the *ywaE*L101E-*tyrZ-lacZ* fusion in the absence of 4-ALP was 3-fold lower in strain EΩ4 than in strain SKO-EΩ4; this is likely to be due to the activity of TyrS in strain EΩ4, which increases the concentration of charged tRNA^{Tyr} and decreases readthrough of the *tyrZ* terminator.

Expression of a *ywaE-tyrZ*Δ*term-lacZ* fusion, in which the 3′ half of the T box terminator helix was deleted, was independent of 4-ALP, consistent with the role of the terminator in the response to tRNA^{Tyr} (Table 3). In addition, basal activity was 20-fold higher in a *ywaE*^{opA7G}*-lacZ* fusion compared to expression of the *ywaE-tyrZ*Δ*term-lacZ* fusion (see Table S3 in the supplemental material). Together, these data demonstrate that both the T box transcriptional terminator and YwaE contribute to regulation of *tyrZ* expression.

Expression of *tyrZ* is required for resistance to D-Tyr. Misincorporation of D-Tyr into proteins inhibits growth (2). Since TyrZ is more selective for L-Tyr *in vitro*, we predicted that expression of *tyrZ* might reduce sensitivity to growth inhibition by D-Tyr. Zone-of-growth-inhibition assays were used to assess growth inhibition by D-Tyr. Growth of strain BR151 was inhibited by D-Tyr in a concentration-dependent manner (data not shown). Inactivation

TABLE 4 Growth inhibition in the presence of D-Tyr

Strain	*tyrS*/*tyrZ*[b]	Radius of zone of growth inhibition (mm)[a]	
		− *dtd*	+ *dtd*
BR151	Both	14 ± 0.3	5.7 ± 0.7
ZKO	*tyrS*	14 ± 0.3	6.0 ± 0.6
SKO-E^{opA7G}	*tyrZ*	7.7 ± 0.3	0
SKO-EΩ4	*tyrZ*	5.3 ± 0.3	0
E^{opA7G}	Both	13 ± 1.2	4.3 ± 0.3
EΩ4	Both	12 ± 0.3	4.7 ± 0.7
3610	Both	3.7 ± 0.3	0
3610-ZKO	*tyrS*	3.7 ± 0.3	0
3610-SKO-E^{V164E}	*tyrZ*	0	0

[a] Bacterial lawns were grown on minimal medium in the presence of filter discs that were soaked with D-Tyr (25 μg). The absence of growth near the filter disc was measured after incubation at 37°C for 24 h. The radius of zone of growth inhibition indicates the size of the clear zone measured from the edge of the filter disc in mm. Values are reported as averages ± standard errors.

[b] Indicates which gene encoding tyrosyl-tRNA synthetase is present in the cell.

of *tyrZ* (resulting in expression of only *tyrS*) in strain ZKO resulted in growth inhibition similar to that of strain BR151 (Table 4), which is consistent with the observation that strain BR151 does not express *tyrZ* at detectable levels (see Fig. S1A in the supplemental material). Strains SKO-E^{opA7G} and SKO-EΩ4, which express only *tyrZ* (see Fig. S1A), showed a reduction in sensitivity to D-Tyr compared to strain BR151. Restoration of the *tyrS* gene in strains EΩ4 and E^{opA7G} resulted in sensitivity to D-Tyr similar to that of the wild-type strain, even though these strains express *tyrZ* (see Fig. S1A), indicating that the presence of TyrS causes sensitivity even in the presence of TyrZ. Growth of the biofilm-forming strain 3610 was sensitive to D-Tyr but was more resistant than that of strain BR151. Inactivation of *tyrZ* in strain 3610-ZKO did not result in increased sensitivity to D-Tyr, which is consistent with observations made for strain BR151. Inactivation of *tyrS* in strain 3610-SKO-E^{V164E} resulted in expression of *tyrZ* (see Fig. S1B) and increased resistance to D-Tyr. These data suggest that expression of *tyrZ* is required for decreased sensitivity to growth inhibition by D-Tyr.

D-Aminoacyl-tRNA deacylase, encoded by the *dtd* gene, removes D-Tyr that has been mischarged onto tRNA^{Tyr}. The *dtd* gene is expressed by most organisms to prevent misincorporation of D-Tyr (2, 9, 10). However, most *B. subtilis* strains contain an inactivated *dtd* gene (3). Since TyrZ is more selective for L-Tyr over D-Tyr, we tested whether *tyrZ* expression provides resistance to growth inhibition by D-Tyr in the presence of a functional *dtd* gene (generated by mutation of the AUU start codon to AUG and inserted into an SPβ prophage for stable introduction into each strain background) (Table 4). Comparison of growth inhibition in the absence of an active *dtd* gene to that in the presence of an active *dtd* gene demonstrated that the *dtd* gene reduced sensitivity to D-Tyr in all strains. Derivatives of strain BR151 that express *tyrS* were still sensitive to D-Tyr. These results suggest that the deacylase reduced but did not eliminate misincorporation of D-Tyr. Growth of strains complemented with the repaired *dtd* gene and expressing only *tyrZ* was not inhibited by D-Tyr at any concentration tested; growth of all strains complemented with the *dtd* gene was not inhibited by D-Tyr at 48 h (data not shown). These data indicate that the *tyrZ* and *dtd* gene products function independently to prevent misincorporation of D-Tyr, consistent with their roles in amino acid selectivity and proofreading, respectively.

The *tyrZ* gene is required for biofilm formation in the presence of D-Tyr. Based on previously published results demonstrating that misincorporation of D-Tyr into cellular proteins indirectly inhibits biofilm formation (3), we tested whether the *tyrZ* gene is important for biofilm formation in the presence of D-Tyr (Fig. 3). Strains 3610, 3610-ZKO, and 3610-SKO-E^{V164E} were grown on biofilm-inducing medium in the presence or absence of D-Tyr. All three strains formed the complex architecture characteristic of pellicle (data not shown) and colony biofilms in the absence of D-Tyr. However, only strain 3610-SKO-E^{V164E}, which expresses *tyrZ* but not *tyrS* (see Fig. S1B in the supplemental material), formed a biofilm in the presence of D-Tyr. The *tyrS* gene was restored in strain 3610-E^{V164E}, in which only the *ywaE* gene was inactivated. This strain was unable to form a biofilm in the presence of D-Tyr (data not shown), indicating that the *ywaE* mutation does not confer biofilm formation in the presence of D-Tyr. These results indicate that expression of *tyrZ* protects against D-Tyr during biofilm formation. Papillae emerged during growth of strains 3610 and 3610-ZKO (in which *tyrZ* has been inactivated) in the presence of D-Tyr, which indicates that these growth conditions result in selection for resistant mutants. The number of papillae was higher in strain 3610-ZKO than in strain 3610 (Fig. 3), suggesting that the presence of the *tyrZ* gene in strain 3610 is partially protective. Complementation with a functional *dtd* gene restored biofilm formation in the presence of D-Tyr, which indicates that expression of the *dtd* gene is sufficient for biofilm formation in the presence of D-Tyr. This is consistent with growth inhibition results since biofilm formation required 72 h and growth of strains complemented with a functional *dtd* gene was

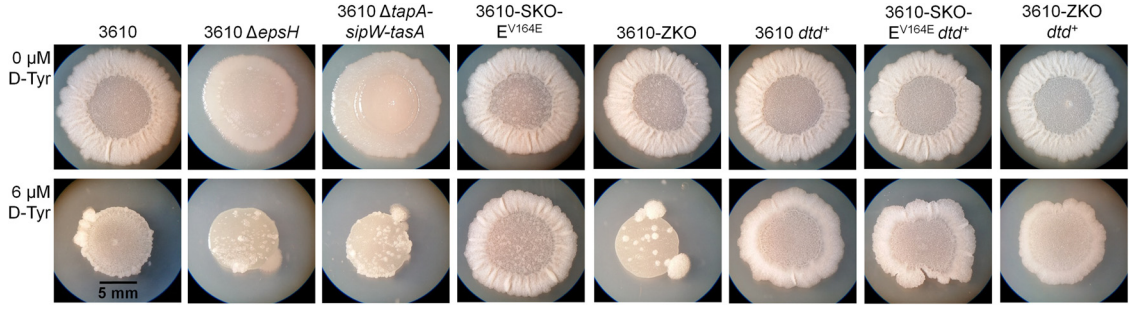

FIG 3 Biofilm formation in the presence or absence of D-Tyr. Overnight cultures (5 μl) were spotted on modified MSgg plates in the presence or absence of D-Tyr (6 μM) and grown at 25°C for 3 days.

not inhibited by D-Tyr after 48 h (data not shown). Biofilm mutant strains 3610Δ*epsH* and 3610Δ*tapA-sipW-tasA* were unable to form a colony or pellicle biofilm in the presence or absence of D-Tyr, as expected (26, 33).

DISCUSSION

The *B. subtilis* genome contains two genes, *tyrS* and *tyrZ*, which encode isoforms of tyrosyl-tRNA synthetase. We found that although TyrZ is less efficient than TyrS at aminoacylation of $tRNA^{Tyr}$ with L-Tyr, it is more selective for L-Tyr over D-Tyr *in vitro*. *B. subtilis* cells that express *tyrZ* alone exhibited decreased sensitivity to D-Tyr *in vivo*, consistent with the selectivity observed *in vitro*. Strains in which both *tyrS* and *tyrZ* are expressed were sensitive to D-Tyr, presumably due to misincorporation of D-Tyr into proteins because of *tyrS* expression. Furthermore, the activity of TyrS is likely to result in reduced transcription of *tyrZ* via the T box mechanism. Therefore, the D-Tyr-sensitive phenotype is dominant to the D-Tyr-resistant phenotype in strains containing both genes.

There are two bacterial subfamilies of tyrosyl-tRNA synthetase (34), represented by TyrS and TyrZ, which in *B. subtilis* show only 30% amino acid identity. Genes in the *tyrZ* class are found in several bacterial phyla, including green sulfur bacteria, CFB group bacteria, *Gammaproteobacteria*, *Deltaproteobacteria*, *Betaproteobacteria*, *Thermotogales*, *Fusobacteria*, *Enterobacteria*, *Aquificales*, and *Firmicutes*. The *tyrZ* gene is the only tyrosyl-tRNA synthetase-encoding gene in *Thermotoga maritima*, *Aquifex aeolicus*, *Thiobacillus ferrooxidans*, and several *Proteobacteria* (e.g., *Burkholderia pseudomallei* and *Pelobacter carbinolicus*). However, there are several organisms in addition to *B. subtilis* that have both *tyrS* and *tyrZ* (e.g., *Pseudomonas aeruginosa*, *Clostridium acetobutylicum*, and *Clostridium botulinum*). The *tyrZ* gene seems to be scattered around the phylogenetic tree, and the presence of *tyrS* and *tyrZ* genes in the same genus makes determining the origin of the *tyrZ* gene unclear.

In *Bacillus* species that have both *tyrS* and *tyrZ* genes, the *ywaE* gene and T box element are also found upstream of *tyrZ*. However, there are instances (e.g., *P. aeruginosa*) where *tyrS* and *tyrZ* are present in a single organism but a transcriptional regulator is not present. There are also examples (e.g., *C. acetobutylicum* and *C. botulinum*) where the *tyrZ* gene contains a T box element but the *tyrS* gene does not.

Our studies clearly demonstrate that TyrZ protects the cell against exogenous D-Tyr during growth and biofilm formation. While it is known that *B. subtilis* produces D-amino acids, including D-Tyr (7, 35, 36), the observation that expression of *tyrZ* did not influence biofilm formation in the absence of D-Tyr suggests that endogenous D-Tyr production is insufficient to inhibit biofilm formation, under the conditions tested.

tyrZ may also protect the cell against misincorporation of other tyrosine analogs. We observed that TyrZ is more selective than TyrS for the activation of meta-tyrosine (*m*-Tyr), phenylalanine, and 3,4-dihydroxyphenylalanine (DOPA)–tyrosine (M. Raina and M. Ibba, unpublished results). However, *m*-Tyr was shown previously to be misincorporated at phenylalanine codons (37), and expression of *tyrZ* did not significantly reduce sensitivity to *m*-Tyr *in vivo* (R. N. Williams-Wagner and T. M. Henkin, unpublished results).

The *ywaE* gene in *B. subtilis* was previously predicted to encode a MarR family transcriptional regulator (13). We show that YwaE represses expression of the *ywaE-tyrZ* operon, probably through interaction with the identified operator sequence. Based on the role of *tyrZ* in resistance to D-Tyr, we propose renaming the *ywaE* gene as *dtrR*, for D-*T*yr *r*esistance *r*egulator.

The *tyrZ* gene is also regulated at the level of transcription attenuation by a $tRNA^{Tyr}$-responsive T box riboswitch, so that *tyrZ* is expressed when the concentration of uncharged $tRNA^{Tyr}$ increases. Expression of *tyrS* also may influence expression of *tyrZ*, presumably by the T box mechanism. Support for this prediction comes from the observation that expression of the *ywaE*L101E-*tyrZ-lacZ* fusion decreased 3-fold in strain EΩ4, which expresses both *tyrS* and *tyrZ*, compared to strain SKO-EΩ4, which expresses only *tyrZ*. The basis for this hierarchical gene regulation is not clear but may be related to differential binding of the $tRNA^{Tyr}$ ligand. Furthermore, TyrZ enzyme activity is inhibited by concentrations of L-Tyr greater than 0.3 mM *in vitro*, which provides a mechanism to inhibit TyrZ when conditions are favorable for TyrS activity. However, the concentration of free L-Tyr in *B. subtilis* cells grown under nitrogen-limiting conditions was reported to be less than 0.1 mM (38), which suggests that cellular concentrations of L-Tyr may not be sufficient to inhibit TyrZ.

For the current study, expression of *tyrZ* required mutations in *dtrR*. However, environmental conditions under which *tyrZ* is expressed in the presence of a functional DtrR have not been identified. MarR regulators often respond to anionic, phenolic compounds (18, 19), and *tyrZ* protected against growth inhibition by D-Tyr. Therefore, it is tempting to speculate that DtrR responds to D-Tyr. We were unable to observe derepression of *dtrR* in the presence of D-Tyr (R. N. Williams-Wagner, F. J. Grundy, and T. M. Henkin, unpublished results), possibly because of the growth conditions tested. It has been proposed previously that MarR in *E. coli* senses copper ions released from damaged membrane proteins (39), which suggests that the signal molecule may not always be a phenolic compound. Furthermore, expression of *tyrZ* but not *dtrR* increases when levels of the extracytoplasmic function sigma factor σ^Y are elevated (40, 41). σ^Y may direct transcription of a gene involved in either transport or biosynthesis of a signal molecule that results in derepression of *tyrZ*. Further investigation of the regulation of *tyrZ* expression will provide additional insight into the physiological role of TyrZ.

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