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BRIEF REPORT

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The low mutational flexibility of the EPSP synthase in *Bacillus subtilis* is due to a higher demand for shikimate pathway intermediates

Inge Schwedt[1,2] | **Kerstin Schöne**[2] | **Maike Eckert**[1] | **Manon Pizzinato**[1] | **Laura Winkler**[2] | **Barbora Knotkova**[3] | **Björn Richts**[3] | **Jann-Louis Hau**[4] | **Julia Steuber**[4] | **Raul Mireles**[5] | **Lianet Noda-Garcia**[5] | **Günter Fritz**[4] | **Carolin Mittelstädt**[2] | **Robert Hertel**[2,6] | **Fabian M. Commichau**[1,2]

[1]FG Molecular Microbiology, Institute for Biology, University of Hohenheim, Stuttgart, Germany

[2]FG Synthetic Microbiology, Institute for Biotechnology, BTU Cottbus-Senftenberg, Senftenberg, Germany

[3]Department of General Microbiology, Institute of Microbiology and Genetics, GZMB, Georg-August University of Göttingen, Göttingen, Germany

[4]FG Cellular Microbiology, Institute of Biology, University of Hohenheim, Stuttgart, Germany

[5]Department of Plant Pathology and Microbiology, Hebrew University, Rehovot, Israel

[6]Department of Genomic and Applied Microbiology, Institute of Microbiology and Genetics, Georg-August University of Göttingen, Göttingen, Germany

Correspondence
Fabian M. Commichau, Universität Hohenheim, FG Molecular Microbiology, Institute for Biology, 70599 Stuttgart, Germanny.
Email: fabian.commichau@uni-hohenheim.de

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Abstract

Glyphosate (GS) inhibits the 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase that is required for aromatic amino acid, folate and quinone biosynthesis in *Bacillus subtilis* and *Escherichia coli*. The inhibition of the EPSP synthase by GS depletes the cell of these metabolites, resulting in cell death. Here, we show that like the laboratory *B. subtilis* strains also environmental and undomesticated isolates adapt to GS by reducing herbicide uptake. Although *B. subtilis* possesses a GS-insensitive EPSP synthase, the enzyme is strongly inhibited by GS in the native environment. Moreover, the *B. subtilis* EPSP synthase mutant was only viable in rich medium containing menaquinone, indicating that the bacteria require a catalytically efficient EPSP synthase under nutrient-poor conditions. The dependency of *B. subtilis* on the EPSP synthase probably limits its evolvability. In contrast, *E. coli* rapidly acquires GS resistance by target modification. However, the evolution of a GS-resistant EPSP synthase under non-selective growth conditions indicates that GS resistance causes fitness costs. Therefore, in both model organisms, the proper function of the EPSP synthase is critical for the cellular viability. This study also revealed that the uptake systems for folate precursors, phenylalanine and tyrosine need to be identified and characterized in *B. subtilis*.

INTRODUCTION

Glyphosate (*N*-(phosphonomethyl)-glycine) (GS) is a non-selective herbicide that is used worldwide in agriculture to control weeds (Franz, 1979; Duke & Powles, 2008). The underlying molecular mechanism causing GS-dependent growth inhibition has been well studied in plants, bacteria and other organisms

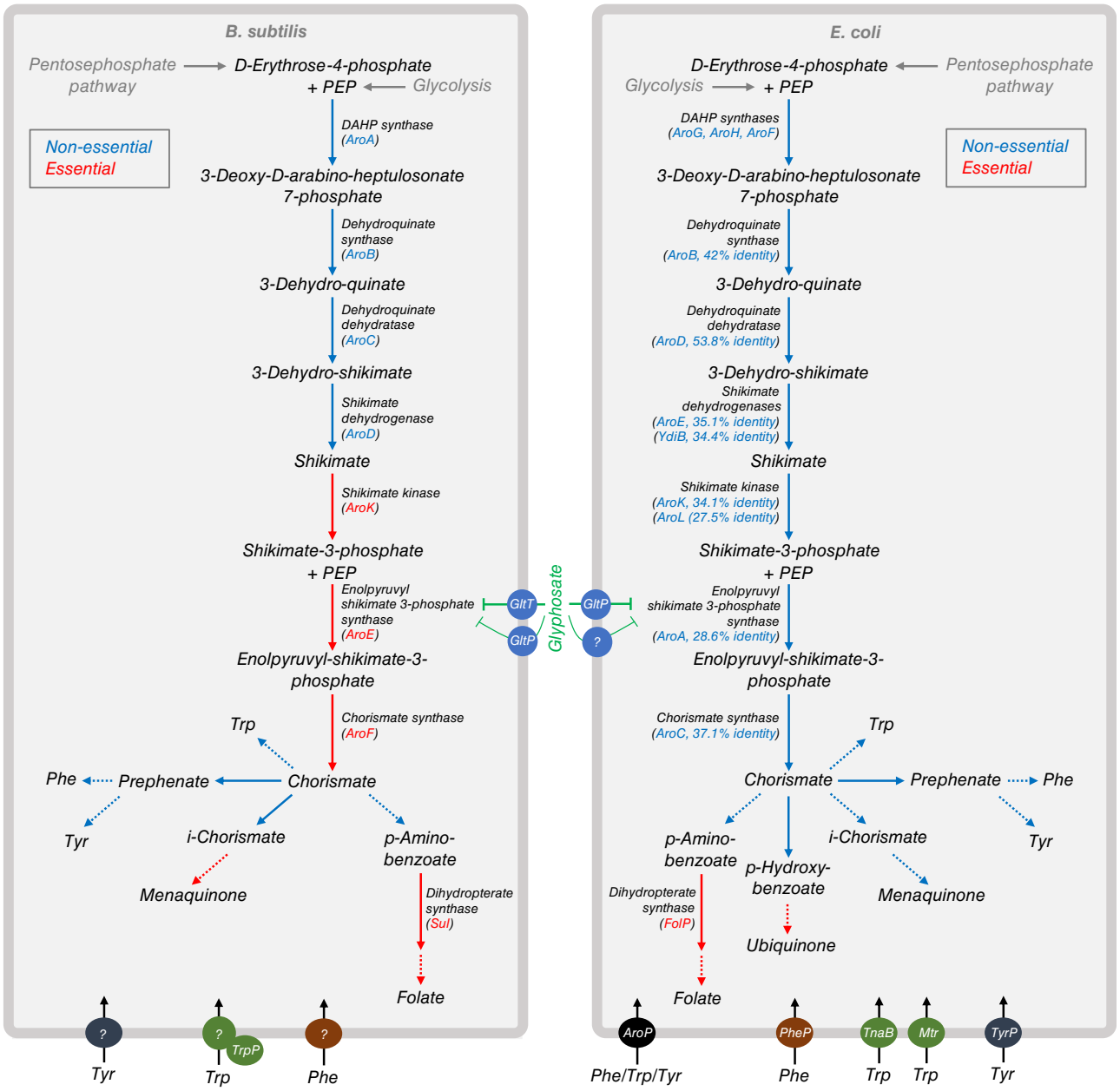

FIGURE 1 Shikimate pathway in *B. subtilis* and *E. coli*. Genes that are essential and not essential for growth in LB medium are highlighted in blue and red, respectively (Baba et al., 2006; Koo et al., 2017). In *B. subtilis*, 6 of 9 and 8 of 15 enzymes are required for MK and folate biosynthesis, respectively, are essential. In *E. coli*, 7 of 9 and 8 of 12 enzymes required for ubiquinone and folate biosynthesis, respectively, are essential. The overall sequence identities between the shikimate pathways enzymes from *B. subtilis* and *E. coli* are shown in the schematic *E. coli* cell. The uptake systems for tyrosine (Tyr) and phenylalanine (Phe) are unknown and the uptake system for tryptophan (Trp) has not been adequately characterized in *B. subtilis*.

(Chekan et al., 2016; Hertel et al., 2021; Pollegioni et al., 2011). GS specifically inhibits the 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase of the shikimate pathway by competing with phosphoenolpyruvate (PEP) for binding to the enzyme (Figure 1; Amrhein et al., 1980; Steinrücken & Amrhein, 1980, 1984; Amrhein et al., 1983; Schönbrunn et al., 2001; Funke et al., 2006). The active EPSP synthase uses PEP and shikimate-3-phosphate to synthesise EPSP, which is converted to chorismate, the precursor for biosynthesis of aromatic amino acids, folate species and quinones (Figure 1; Wilson et al., 1998; Herrmann & Weaver, 1999). Unless provided by the environment, the aromatic amino acids must be synthesized *de novo* as essential protein building blocks. Moreover, folate species including tetrahydrofolate are essential cofactors required for the biosynthesis of purines, pyrimidines, amino acids, pantothenate and *N*-formyl-methionyl-tRNA. Quinones are also essential molecules that serve as electron carriers in the respiratory

chain (Bentley & Meganathan, 1982). The most studied and widespread quinones are ubiquinone (UQ), menaquinone (MK) and 2-demethylmenaquinone (DMK) (Collins & Jones, 1981). While *Bacillus subtilis* only synthesizes MK, bacteria like *Escherichia coli* produce UQ, MK and DMK (Bentley & Meganathan, 1982; Collins & Jones, 1981; Nitzschke & Bettenbrock, 2018). Given the importance of the shikimate pathway in the generation of the precursor chorismate, it is not surprising that the GS-dependent inhibition of the EPSP synthase severely impairs cell viability. In fact, the enzymes of the shikimate, quinone and folate biosynthesis pathways are attractive targets for novel antibiotics (Brilisauer et al., 2019; Frlan, 2022).

The intensive use of GS in agriculture has led to the development of resistance to the herbicide in bacteria, fungi and plants (Funke et al., 2006; Powles, 2008; Shaner et al., 2012; Sammons & Gaines, 2014; Chekan et al., 2016; Hertel et al., 2021; Molina-Santiago & Udaondo, 2022; Patriarcheas et al., 2023). For instance, the modification of the GS target through the acquisition of mutations in the EPSP synthase gene is a widespread resistance mechanism (Barry et al., 1992; Chekan et al., 2016; Comai et al., 1983; Della-Cioppa et al., 1987). Moreover, organisms can develop GS resistance due to mutations resulting in overproduction of the EPSP synthase or PEP, the co-substrate of the enzyme (Gaines et al., 2010; Dillon et al., 2017; Wicke et al., 2019; Schwedt et al., 2023). The ability of bacteria to degrade GS also confers resistance to the herbicide (Hove-Jensen et al., 2014; Hertel et al., 2021). Furthermore, GS can be detoxified by covalent modification (Rao et al., 1983; Penaloza-Vazquez et al., 1995; Castle et al., 2004). Finally, altered GS transport may increase resistance to the herbicide (Hertel et al., 2022; Liebrenz et al., 2022; Rong-Mullis et al., 2017; Staub et al., 2012; Tao et al., 2017; Wicke et al., 2019).

We are interested in the evolution of GS resistance in bacteria. Previously, we have observed that the *B. subtilis* laboratory strain 168 exclusively develops GS resistance by the acquisition of loss-of-function mutations in the *gltT* and *gltP* genes encoding the high- and low-affinity glutamate transporters GltT and GltP, respectively (Figure 1; Tolner et al., 1995; Zaprasis et al., 2015; Wicke et al., 2019). Like GS, uptake of the 3-dehydroquinate synthase inhibitor 7-deoxysedoheptulose is also mediated by promiscuous transporters (Rapp et al., 2021; Wicke et al., 2019). In contrast to *B. subtilis*, amino acid substitutions in the EPSP synthase or enhanced synthesis of the enzyme allow *E. coli* to grow in the presence of otherwise toxic levels of GS (Wicke et al., 2019).

Here, we show that the undomesticated *B. subtilis* strain NCIB3610, a close relative of laboratory strain 168, and environmental *Bacillus* isolates, evolve GS resistance by mutational inactivation of the *gltT* glutamate transporter gene. A suppressor screen with a *B. subtilis* strain synthesizing a C-terminally truncated EPSP synthase variant revealed that the enzymatic reaction cannot be bypassed by enhancing the uptake of aromatic amino acids or by recruiting a promiscuous enzyme. In contrast to *E. coli*, the EPSP synthase activity is essential for growth of *B. subtilis* in lysogeny broth (LB) but not in even richer brain-heart infusion. Moreover, MK allowed growth of the *B. subtilis* EPSP synthase mutant in LB medium, showing the requirement for MK precursors. Thus, the nutritional requirements of the EPSP synthase-deficient *B. subtilis* mutant are more complex than those of *E. coli*. Furthermore, we found that the *B. subtilis* EPSP synthase is less sensitive to GS than the enzyme from *E. coli*. This fundamental difference in the dependence of the two model bacteria on the shikimate pathway could explain why the *E. coli* EPSP synthase but not the counterpart from *B. subtilis* tolerates amino acid substitutions reducing GS sensitivity. Finally, we observed that an *aroA* allele conferring GS resistance in *E. coli* continuously evolves under non-selective growth conditions, indicating that GS resistance causes substantial fitness costs.

EXPERIMENTAL PROCEDURES

Bacterial strains, chemicals and DNA manipulation

Bacteria used in this study are listed in Table S1. Primers were purchased from Sigma-Aldrich (Munich, Germany) and are listed in Table S2. Chemicals and media were purchased from Sigma-Aldrich (Munich, Germany), Carl Roth (Karlsruhe, Germany) and Becton Dickinson (Heidelberg, Germany). Bacterial chromosomal DNA was isolated using the peqGOLD bacterial DNA kit (Peqlab, Erlangen, Germany). PCR products were purified using the PCR purification kit (Qiagen, Germany). Phusion DNA polymerase was purchased from Thermo Scientific (Germany) and used according to the manufacturer's instructions.

Cultivation of bacteria

Bacteria were grown in lysogeny broth (LB) (Sezonov et al., 2007), brain hear infusion (BHI), SP, M9 (Miller, 1972) and C-Glc medium (Commichau et al., 2007; Dormeyer et al., 2019). C-Glc medium contain glucose and ammonium as carbon and nitrogen sources, respectively. CS-Glc medium contains succinate as an additional source of carbon (Commichau et al., 2007). Agar plates were prepared with 15 g agar/l (Roth, Germany). Growth in liquid medium was monitored using 96-well plates (Microtest Plate 96-Well, F Sarstedt, Germany) at 37°C and medium orbital

TABLE 2 Isolated *aroA* alleles.

Plasmid	*aroA* allele	Mutation(s)[a]	Effect on protein	Active	Reference
aroA allele with a single mutation					
pBP668	GS insensitive	**C358A**	R120S	+	R120S substitution described in Wicke et al., 2019
aroA alleles with two or more mutations					
pBP649	*aroA* C358A derivative	**C358A** C510T	R120S	+	-
pBP651	*aroA* C358A derivative	**C358A** T732C	R120S	+	-
pBP654	*aroA* C358A derivative	**C358A** A1200T	R120S	+	-
pBP661	*aroA* C358A derivative	**C358A** T492C	R120S	+	-
pBP673	*aroA* C358A derivative	**C358A** T457C	R120S	+	-
pBP681	*aroA* C358A derivative	A312G **C358A**	R120S	+	-
pBP666	*aroA* C358A derivative	**A266G C358A**	E89G R120S	+	-
pBP677	*aroA* C358A derivative	**C358A T398C**	L133P R120S	–	-
pBP658	*aroA* C358A derivative	**C358A T442C**	R120S Y148H	+	-
pBP648	*aroA* C358A derivative	**C358A T716C**	R120S V239A	+	-
pBP663	*aroA* C358A derivative	**C358A G829A**	R120S A277T	+	-
pBP670	*aroA* C358A derivative	**C358A T902C**	R120S L301P	–	-
pBP684	*aroA* C358A derivative	**C358A C1198T**	R120S P400S	+	-
pBP680	*aroA* C358A derivative	**C358A C1205T**	R120S T402M	+	-
pBP690	*aroA* C358A derivative	**T620A T1103C**	R120S L207Q I368T	+	-
pBP653	*aroA* C358A derivative	T72G T300C **C358A**	R120S	+	-
pBP669	*aroA* C358A derivative	**C358A** A525G A1053G	R120S	+	-
pBP665	*aroA* C358A derivative	**A295G** A255G **C358A**	M99V R120S	+	-
pBP657	*aroA* C358A derivative	**C358A** C672T **T881C**	R120S I294T	+	-
pBP644	*aroA* C358A derivative	**C358A A805G G849C**	R120S S269G M283I	+	-
pBP667	*aroA* C358A derivative	**C358A G829A T1237C**	R120S A277T F413I	+	-
pBP675	*aroA* C358A derivative	**C358A T1187C A1234G**	R120S L396S T412A	–	-
pBP689	*aroA* C358A derivative	**A289G C358A** T1014C **T1172C**	T97A R120S F391S	–	Single T97I substitution results in decrease of PEP affinity (Funke et al., 2009)
pBP687	*aroA* C358A derivative	**A137G C358A** C483T **T1202C**	D46G R120S V401A	+	-
pBP647	*aroA* C358A derivative	**A266G C358A A592G A626G**	E89K R120S K198E K209R	+	-

(Continues)

TABLE 2 (Continued)

Plasmid	*aroA* allele	Mutation(s)[a]	Effect on protein	Active	Reference
pBP656	*aroA* C358A derivative	**C176T A206G C358A T1178A**	A59V D69G R120S L393Q	+	-
pBP655	*aroA* C358A derivative	G135A **C358A A361G** G552A T882C	R120S M121V	+	-
pBP688	*aroA* C358A derivative	**A102T G109A C260T C358A** G990A	L34F G37S A87V R120S	+	-
pBP659	*aroA* C358A derivative	**C358A G721A T730C T1181C** G754A	R120S G241S S244P A252T I294A	–	
pBP686	*aroA* C358A derivative	**A67G** T100C **C358A A641G T968C A1135G** T1149C	S23G R120S E214G L323S I379V	–	-
pBP674	*aroA* C358A derivative	**C42T C358A** C403T T519C **A938G A1141G T1166C A1255T**	G14V R120S D313G T381A M389T T409S	–	D313A inactives the enzyme (Eschenburg et al., 2003)
pBP676	*aroA* C358A derivative	T88C **C358A T377C** G549C **A625G** A759G **A781G T877G T902A**	R120S I126T K209E K261E Y293D L301Q	–	-
pBP664	*aroA* C358A derivative	**A64G A65G A128G C311T C358A G410A A443G**	K22G N43S A104V R120S G137E Y148C	–	K22 required for binding of shikimate-3-phosphate (Priestman et al., 2005)
aroA alleles with nonsense mutations					
pBP650	*aroA* C358A derivative	**A191G C358A C446T T1187A**	Y64C R120S P149L Δ33 a C terminus	–	-
pBP662	*aroA* C358A derivative	**T122C Δ546T**	L41S Δ240 a C terminus	–	-
pBP671	*aroA* C358A derivative	**ΔC19 ΔT456 A421T A435G T668C A842C**	Q7N Δ394 aa C-terminus	–	-
pBP685	*aroA* C358A derivative	**C358A A565G ΔC1280**	R120S I189V A427 + E429	–	-

[a]Mutations that affect the protein sequence are shown in bold letters.

shaking at 237 cpm (4 mm) in a Synergy H1 plate reader (Agilent, USA) equipped with the Gen5 software, and the OD_{600} was measured in 10–15 min intervals. Single colonies were used to inoculate 5 mL overnight LB cultures that were incubated at 220 rpm and 30°C. The OD_{600} was adjusted to 0.1 and 150 μL of the cell suspensions were transferred into 96-well plates. Bacteria were cultivated in the Synergy H1 plate reader as described above.

Plasmid construction

The plasmids used in this study are listed in Table S3. The plasmids pBP759 and pBP765 carrying the *B. subtilis aroE* ΔA1256 and *aroE* wild type alleles, respectively, were constructed as follows. The *aroE* gene was amplified by PCR using the primer pair iGEM2018_27/BR126. The primer iGEM2018_27 contains the Shine-Dalgarno sequence of the *B. subtilis gapA* (Table S3). The PCR product was digested with *Bam*HI and *Sal*I and ligated with pBQ200 (Martin-Verstraete et al., 1994) that was digested with the same enzymes. DNA sequencing of several pBQ200::*aroE* clones led to the isolation of the plasmid pBP756 containing the wild type *aroE* gene and the pBP759 (*aroE* ΔA1256) encoding the truncated EPSP synthase. The plasmids pBP649–pBP690 carrying the *E. coli aroA* mutant alleles (Table 2) were constructed as follows. The *aroA* alleles were amplified by PCR using the primer pair DW7/DW8 and cell material from a single colony of the *E. coli* strain BPE1 (*aroA* C358A) containing chromosomal material as the template DNA. The PCR product was digested with *Bam*HI and *Sal*I and ligated with pBQ200 that was digested with the same enzymes. All generated plasmids were verified by Sanger sequencing (Microsynth-SeqLab Sequence Laboratories).

Isolation of spore-forming Bacillus isolates

The soil sample, which possibly contained spore-forming *Bacillus* isolates, was taken from a field edge in Dresden (Ockwitzer Allee 20, 01156 Dresden, Germany). Before further treatment, the sample was stored in a cool and dry place for 3 weeks. 1 g of the soil sample was mixed with 99 mL of water, shaken for

1 min and 1 mL of the mixture was heated in a boiling water bath to kill the vegetative cells. The boiled sample was 1000-fold diluted and 100 μL of the 10^{-2} and 10^{-3} dilutions were propagated on FP rich medium agar plates (Sifin diagnostics). The plates were incubated for 7 days at 28°C. Dull, non-mucoid colonies were identified, the rod-shaped cell morphology was verified under the light microscope and colony material was propagated on FP plates that were incubated for 7 days at 28°C. The Gram-positive 'phenotype' of 22 isolates was verified by a Gram-staining and the isolates were further cultivated on sporulation (SP) agar plates (8 g/L nutrient broth, 0.25 g/L $MgSO_4 \times 7\ H_2O$, 1 g/L KCl, 0.5 M $CaCl_2$, 10 mM $MnCl_2$, 4.4 mg/L ferric ammonium citrate, 15 g/L bacto agar; Table S4).

Taxonomic classification of bacteria

The initial taxonomic classification of the bacteria that were isolated from the soil samples was realized by sequencing of the 16S rRNA genes. The 16S rRNA genes were amplified by PCR using the primer pair 27F/1492R (Frederikson et al., 2013). The sequences obtained by Sanger sequencing (Microsynth-SeqLab Sequence Laboratories) were evaluated using the Geneious software package (Kearse et al., 2012) and compared to the rRNA/ITS databases of NCBI via BLASTn (Table S5). Furthermore, both draft genome sequence data sets were uploaded to the Type (Strain) Genome Server (TYGS), available at https://tygs.dsmz.de, for a whole genome-based taxonomic analysis (Meier-Kolthoff & Göker, 2019). All genomes of type strains involved in the TYGS analysis were downloaded from GenBank in FASTA format and used for average nucleotide identity analysis using the average_nucleotide_identity.py script (https://github.com/widdowquinn/pyani#script-average_nucleotide_identitypy), employing the ANIm option.

Disc diffusion assay

The strains were grown overnight in LB and next day, the cells were washed twice in 1× C-salt solution (4 g/L KH_2PO_4, 16 g/l K_2HPO_4, 1.3 g/L $(NH_4)_2SO_4$) and the OD_{600} was adjusted to 1.0. On C-Glc plates, 100 μL of the diluted cells suspension were propagated. A total of 10 μL of the herbicide solutions (glufosinate, 1 M; Roundup®, 360 g/L glyphosate isopropylamine salt) were added to a paper disc that was placed in the center of the plate. The plates were incubated for 24 h at 37°C.

Genome sequencing

Genomic DNA was prepared from 500 μL overnight cultures using the MasterPure Complete DNA & RNA Purification Kit (Lucigen, Middleton, USA) following the instruction of the manufacturer with the modification of physically opening cells with the TissueLyser II (Qiagen). Purified genomic DNA was paired end sequenced (2 × 150 bp) (GENEWIZ). Sequence libraries were prepared with the NEBNext Ultra II FS DNA Library Prep 136 kit (New England Biolabs GmbH, Frankfurt, Germany) and sequenced with the NovaSeq6000 (Illumina, San Diego, CA, USA). Raw-read quality was assessed with FastQC version 0.11.9 (Andrews, 2010) and quality processed using Trimmomatic v.0.36 (Bolger et al., 2014). SPAdes version 3.14.0 with the options—*only-assembler* and—*isolate* was used for genome assembly. QualiMap v.2.2.1 (Okonechnikov et al., 2016), which employed bowtie2 (v.2.2.6) (Langmead & Salzberg, 2012) was used to verify assembly quality and sequence coverage. Contigs <500 bp and less than 10% of the average coverage were removed. The Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016) performed auto-annotation of the genomes during genome submission to GenBank (Benson et al., 2013). Single-nucleotide polymorphisms were identified via the Breseq pipeline (Deatherage & Barrick, 2014). Annotated draft genomes from the isolates 3, 17 and 18 (GCF_021728915.1, GCF_021728925.1, GCF_021728955.1) served as reference and raw sequences (PRJNA800220, PRJNA800222, PRJNA800225) from the GS-resistant mutants as input material. Sequencing reads obtained with the chromosomal DNAs of the *B. subtilis aroE* mutant BP250 and *E. coli aroA* mutant JW0891-1 were analysed using the Geneious software package (Kearse et al., 2012).

Preparation for scanning electron microscopy

The environmental isolates 3, 17 and 18 were grown overnight at 37°C in 4 mL LB. Next day, the cells diluted 1:100 with 10 mL LB medium in 100 mL shake flasks supplemented and the cultures were grown at 37°C until an OD_{600} of about 0.8. The cells of 1 mL of the cultures were harvested by centrifugation for 2 min and 2,000 g at room temperature. The supernatants were discarded, the cell pellets were resuspended in 1 mL 4% (w/v) paraformaldehyde/PBS solution (pH 7.4) and the fixation of the cells occurred for 2 h at room temperature under rigorous shaking. The cells were pelleted, resuspended in 1 mL 8% (w/v) paraformaldehyde/PBS solution (pH 7.4) and incubated over night at 4°C under agitation. The fixation solution was removed by centrifugation and the cells were washed twice in 1 mL PBS. Next, the cells were dehydrated by stepwise incubation for 15 min with 30%, 50%, 70%, 90% and for 30 min with 100 % (v/v) ethanol. The cells were collected by centrifugation, the supernatants were

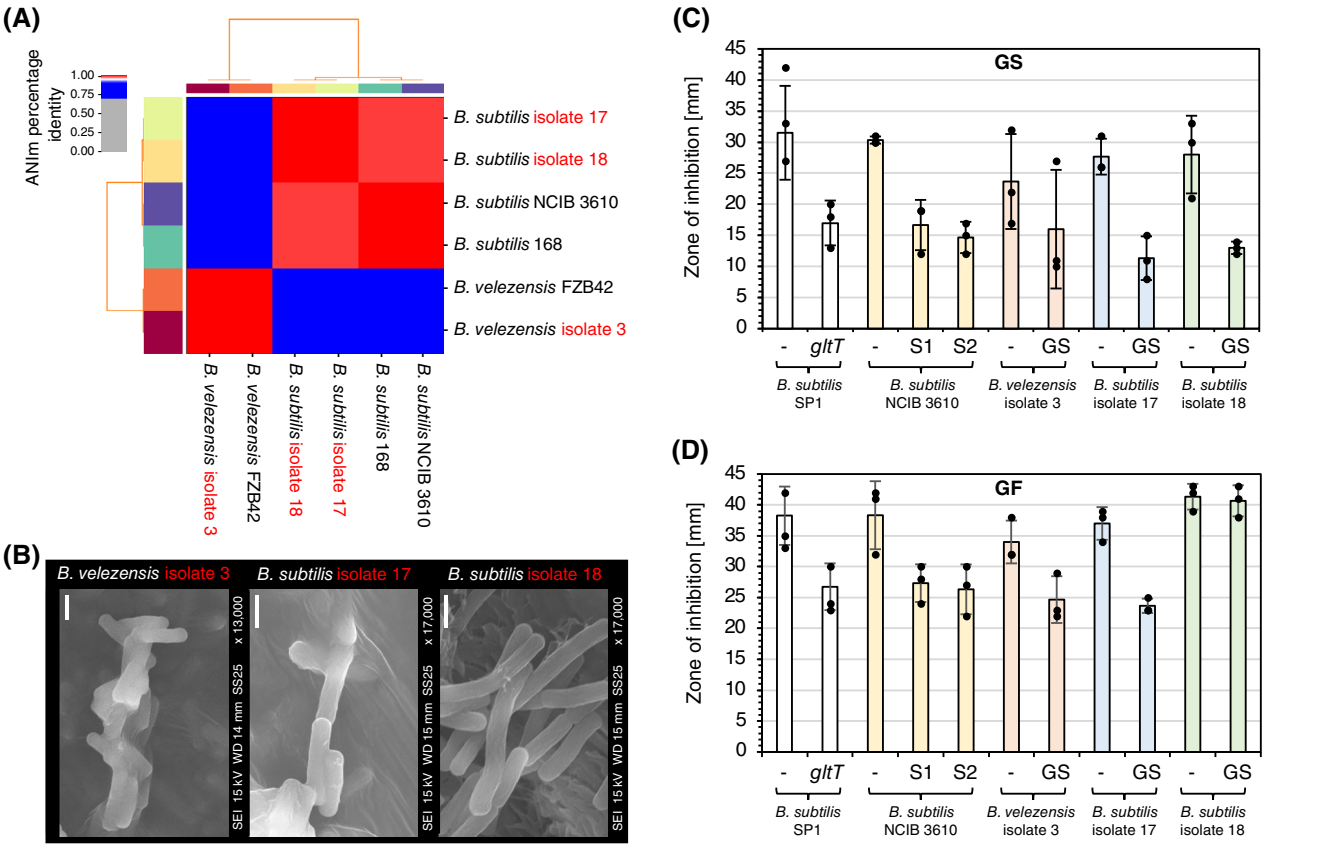

FIGURE 2 Phylogenetic analysis, characterization, and herbicide susceptibility of environmental *Bacillus* isolates. (A) For the phylogenetic analysis, the indicated strains from the genus *Bacillus* were considered. Calculations were done with pyani (https://github.com/widdowquinn/pyani; Richter & Rosselló-Móra, 2009; Arahal, 2014). Using the ANIm method with standard parameters. (B) Scanning electron microscopic analyses of environmental *Bacillus* strains. Magnifications: *B. velezensis* isolate 3, ×13,000; *B. subtilis* isolate 17, ×17,000; *B. subtilis* isolate 18, ×17,000. (C, D) GS and GF susceptibility tests. The *B. subtilis* laboratory strain SP1 and the *gltT* mutant iGEM41 served as controls. Error bars represent standard errors of means of triplicate plating experiments.

removed, and the residual ethanol evaporated in a SpeedVac. The cells were transferred onto a stamp for scanning electron microscopy, gold sputter coated and analysed by microscopy.

RESULTS

Evolution of GS resistance in the undomesticated *B. subtilis* strain NCIB 3610

The laboratory *B. subtilis* strain 168 exclusively acquires GS resistance by the mutational inactivation of the *gltT* and *gltP* glutamate transporter genes (Wicke et al., 2019). To assess which mutations confer GS resistance to the undomesticated *B. subtilis* strain NCIB 3610, we isolated eleven mutants that appeared on C-Glc minimal medium agar plates that contained 10 mM GS and were incubated for 48 h at 37°C. Next, we analysed the resistance of the arbitrarily chosen GS suppressors S1 and S2 to glufosinate (GF), which inhibits the glutamine synthetase (Bayer et al., 1972), by performing a disc diffusion assay. The laboratory *B. subtilis* strain SP1 and the *gltT* mutant served as a control. Since GF also enters the *B. subtilis* cell *via* the glutamate transporter GltT, increased GF resistance would be indicative for the inactivation of the *gltT* gene (Wicke et al., 2019). As shown in Figure 2C,D, the NCIB 3610-derived GS suppressors S1 and S2 were both resistant to GS and GF, suggesting that *gltT* was indeed inactivated. Subsequent Sanger sequencing analyses confirmed that all NCIB 3610-derived GS suppressors had acquired mutations in the *gltT* gene (Table 1). Seven suppressors had acquired deletions and insertions in the *gltT* gene that would either truncate or alter the predicted membrane topology of GltT (Table 1; Figure S1). In four GS suppressors, amino acid substitutions in the transmembrane (TM) helix 7 and in the extracellular loop between TM helices 9 and 10 of GltT likely reduce its transporter activity (Figure S1). To conclude, like the laboratory *B. subtilis* strain 168 also the undomesticated strain NCIB 3610 acquires GS resistance by mutating the *gltT* glutamate transporter gene.

TABLE 1 GS-resistant *Bacillus* mutants.

GS-resistant mutants derived from the *B. subtilis* strain NCIB 3610			
Mutant	**Strain**	**Affected gene, mutations**	**Amino acid exchanges, effect on the protein**
S1	BP1325	*gltT*, G1144T A1290G	D382Y, probably less active
S2	BP1326	*gltT*, ΔC29-T707	C-terminally truncated by 419 amino acids, inactive
S5	BP1327	*gltT*, +T538	C-terminally truncated by 248 amino acids, inactive
S6	BP1328	*gltT*, +A80-A202	Duplication of 41 amino acids (+N29-69G), altered membrane topology
S7	BP1329	*gltT*, +C457 G863A	C-terminally truncated by 258 amino acids, inactive
S9	BP1330	*gltT*, +A842	C-terminally truncated by 126 amino acids
S10	BP1331	*gltT*, ΔC231-T278	Lack of 16 amino acids (-R78-L93), altered membrane topology
S11	BP1332	*gltT*, A90C ΔC92 ΔA842	C-terminally truncated by 384 amino acids
S12	BP1333	*gltT*, G863A	C288Y, probably less active
S13	BP1334	*gltT*, G863A	C288Y, probably less active
S14	BP1335	*gltT*, G863A	C288Y, probably less active
GS-resistant mutants derived from the *Bacillus* isolates 3, 17 and 18			
Mutant	**Strain**	**Affected gene, mutations**	**Amino acid exchanges, effect on the protein**
GS3	BP1320	*gltT*, ΔA910-T913	C-terminally truncated by 126 amino acids, inactive
GS17	BP1321	*gltT*, A1204T	C-terminally truncated by 346 amino acids, inactive
GS18	BP1322	*gltT*, G1033C	G345R, probably less active
GF1[a]	BP1323	*gltT*, +G55	C-terminally truncated by 346 amino acids, inactive
GF2[a]	BP1324	*gltT*, ΔC127	C-terminally truncated by 382 amino acids, inactive

[a]The GF-resistant mutants are derived from the *B. velezensis* isolate 3.

Evolution of GS resistance in environmental *Bacillus* isolates

Next, we assessed the GS resistance of environmental spore-forming *Bacillus* isolates and their potential to evolve GS resistance. For this purpose, we isolated 22 strains from a soil sample (Table S4). Initial taxonomic classification by 16S rRNA gene sequencing analysis revealed that 21 isolates belong to the genus *Bacillus* (Table S5). Next, we analysed the growth of the isolates on C-Glc plates, which is a prerequisite for investigating GS resistance. Previously, it has been shown that the growth medium must be free of amino acids like glutamate and aspartate because they interfere with the uptake of GS *via* glutamate transporters in *B. subtilis* (Wicke et al., 2019; Schwedt et al., 2023). We continued to work with 20 of the 22 isolates that could be cultivated on minimal medium plates (Table S4). On C-Glc plates with 5 mM GS, 9 of the 20 isolates grew, while 4 isolates grew even in the presence of 10 mM GS (Table S4). On C-Glc plates with 10 mM GS, 7 isolates formed suppressor mutants, among them 4 that grew in the presence of 5 mM GS (Table S4). Next, we isolated single suppressor mutants derived from the arbitrarily selected isolates 3, 17 and 18. A comparison of the genome sequences of the isolates 3, 17 and 18 with that of phylogenetically relates species using the Type (Strain) Genome Server (TYGS) revealed that the isolate 3 associates with *Bacillus velezensis* FZB42 and isolates 17 and 18 with the *B. subtilis* strains 168 and NCIB 3610, respectively (Meier-Kolthoff & Göker, 2019; Figure 2C). Therefore, the isolates 3, 17 and 18 were designated as *B. velezensis* isolate 3, *B. subtilis* isolate 17 and *B. subtilis* isolate 18, respectively. Scanning electron microscopy analyses revealed that the isolates form rod-shaped cells, which may be embedded in an extracellular matrix (Figure 2D). To assess GS and GF resistance of *B. velezensis* isolate 3, *B. subtilis* isolate 17 and *B. subtilis* isolate 18 and their suppressors GS3, GS17 and GS18, we performed disc diffusion assays. As shown in Figure 2C,D, the three suppressors showed increased GS resistance and GF resistance of GS3 and GS17 was also enhanced. This suggests that a transporter gene was indeed inactivated in the evolved strains. To identify the mutations that confer resistance against GS, we performed genome sequencing with the isolates 3, 17 and 18 and their suppressors, and compared the genomes. The suppressors GS3 and GS17 had acquired deletions and a single nucleotide exchange, respectively, in the *gltT* gene that would truncate the GltT protein (Table 1). In suppressor GS18, the amino acid substitution G345R in the intracellular loop between TM helices 8 and 9 of GltT selectively reduces GS transport activity (Figure S1). Therefore, a suppressor screen based on

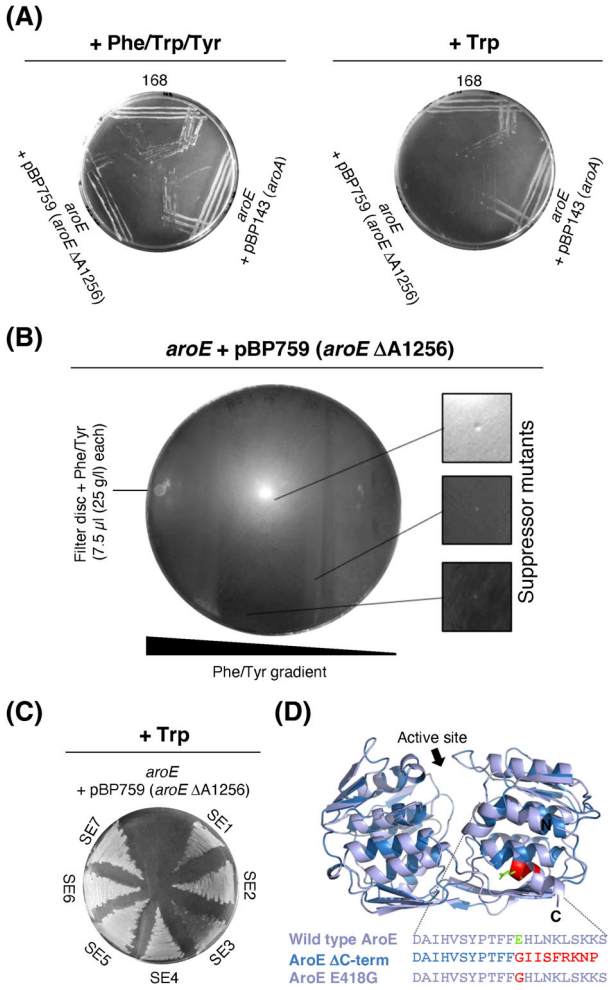

FIGURE 3 Suppressor analysis with a C-terminally truncated EPSP synthase in *B. subtilis*. (A) Growth of the *B. subitilis* laboratory strain 168 and of the *aroE* mutant expressing either the *E. coli aroA* gene or the *aroE* ΔA1256 mutant allele on CS-Glc plates. The amino acids phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr) were added to a final concentration of 245 µM. The agar plates were incubated for 24 h at 37°C. (B) Isolation of suppressor mutants derived from the *aroE* mutant carrying the plasmid pBP759 (*aroE* ΔA1256). The agar plate was incubated for 5 days at 37°C. (C) Growth of 7 selected suppressor mutants (SE1–SE7) that were isolated from the plate shown in B. The parental strain *aroE* + pBP759 (*aroE* ΔA1256) served as a control. The CS-Glc plate contained 245 µM Trp. The agar plate was incubated for 24 h at 37°C. (D) Structure models for *B. subtilis* AroE, AroE ΔC-term (truncated by one amino acid) and AroE E418G that emerged in the suppressor screen. The AroE models were generated using the SWISS-Model server for homology modelling of protein structures (Waterhouse et al., 2018) and a model of the structure of the EPSP synthase from *Bacillus halodurans* C-125 (PDBid: 3RMT).

GS resistance could be useful to isolate additional GltT variants that discriminate between GS and GF as the substrates. We also observed the emergence of GF-resistant mutants on the disc diffusion assay plate that contained the *Bacillus* isolate 3. Sanger sequencing revealed that the suppressors GF1 and GF2 had acquired insertions and deletions, respectively, in the *gltT* gene that would truncate GltT. Thus, at least for isolate 3, the selection for GF resistance results in the inactivation of the promiscuous GltT glutamate transporter. To conclude, unlike *E. coli*, the *B. subtilis* laboratory strains 168 and NCIB 3610 as well as environmental *Bacillus* isolates acquire GS resistance by mutating the *gltT* glutamate transporter gene.

A functional EPSP synthase is required for growth of *B. subtilis* in minimal medium

Bacteria can adapt to the limitation of essential substances by the acquisition of beneficial mutations (Commichau et al., 2015; Gundlach, Herzberg, Kaever, et al., 2017; Gundlach, Herzberg, Hertel, et al., 2017; Richts et al., 2021). The genomic alterations in turn may alter the activities of uptake systems and affect central metabolism thereby allowing the bacteria to cope with these limitations. Recently, it has indeed been shown that instead of improving a needed enzyme activity by the accumulation of mutations in the coding gene, most adaptive mutations occurred elsewhere in the genome (Morgenthaler et al., 2019). To assess whether spontaneous genomic mutations may compensate for reduced EPSP synthase activity in the background of a *B. subtilis aroE* mutant, we performed a suppressor screen with the strain *aroE*-pBP759. The strain carries the plasmid pBP759 (*aroE* ΔA1256) that codes for the EPSP synthase variant AroE ΔC-term with reduced activity due to a truncation and altered amino acid sequence at the C-terminus (Figure 3D). The plasmid was obtained incidentally during the construction of the plasmid pBP765 (*aroE*), which can be used for complementation studies. For the strain construction, we introduced the plasmids pBP143 and pBP759 encoding the *E. coli* EPSP synthase AroA and AroE ΔC-term, respectively, into the *B. subtilis* strain 168 and deleted the chromosomal *aroE* copy using a deletion cassette that contains a spectinomycin resistance gene as described previously (Wicke et al., 2019). Unlike the control strain *aroE*-pBP143, the *aroE* mutant producing the AroE ΔC-term variant only grew on minimal medium plates supplemented with all aromatic amino acids (Figure 3A). Next, we aimed to isolate extragenic suppressor mutants that gained the ability to grow on minimal medium despite the presence of a catalytically inefficient EPSP synthase. For this purpose, we propagated the strain *aroE*-pBP759 (*aroE* ΔA1256) on C-Glc minimal medium plates containing tryptophan that is required by the bacteria due to the *trpC2* mutation. To obtain a phenylalanine and tyrosine gradient that could facilitate the isolation of suppressor mutants, we placed a filter disc containing both amino acids at the edge of the agar plate (Figure 3B). After incubating the plate for five days at 37°C, several suppressor mutants emerged in the centre of the plate

(Figure 3B). Seven randomly selected suppressor mutants were isolated and their ability to grow independently of phenylalanine and tyrosine was confirmed using minimal medium plates (Figure 3C). To identify the mutations allowing the phenylalanine/tyrosine-independent growth, we performed genome sequencing analyses with the cDNAs of suppressors 1 and 2. In both suppressor mutants, the insertion of the nucleotide G at position 1256 in the plasmid-encoded *aroE* gene leads to amino acid substitution E418G and restoration of the remaining C-terminal protein sequence (Figure 3D). Sanger sequencing analyses revealed that the *aroE* alleles of the suppressor mutants SE3−SE7 also carried the G1256 insertion. The plasmid containing the *aroE* A1256G allele was designated as pBP764. To conclude, *B. subtilis* requires a functional EPSP synthase for rapid growth in minimal medium. Since all isolated suppressor mutants had acquired intragenic mutations, it is rather unlikely that genomic alterations may allow *B. subtilis* to grow independently of a fully functional EPSP synthase (see below).

The EPSP synthase is dispensable for growth of *B. subtilis* in nutrient rich medium

Previously, we have shown that the *aroE* gene is essential for growth of *B. subtilis* in lysogeny broth (LB) (Wicke et al., 2019). The essentiality of the EPSP synthase in *B. subtilis* probably explains its low mutational flexibility. It is tempting to speculate that amino substitutions in AroE would interfere with its catalytic activity, thus affecting the synthesis of precursors for the biosynthesis of the aromatic amino acids as well as of folates and menaquinone. To assess whether the *aroE* gene is dispensable for growth in nutrient rich brain heart infusion (BHI), we transformed the *B. subtilis* wild type strain SP1 with DNA that was obtained from the strain *aroE*-pBP759. In this strain, the chromosomal *aroE* copy was replaced by a spectinomycin resistance gene and the *aroE* ΔA1256 allele is expressed from the plasmid pBP759 that confers resistance against erythromycin/lincomycin (Wicke et al., 2019). Several large and small colonies emerged on the transformation plates only containing spectinomycin. Replica plating revealed that the large colonies were resistant against spectinomycin and erythromycin/lincomycin, indicating the deletion of the *aroE* gene and the presence of the plasmid pBP759. In contrast, the small transformants were only resistant to spectinomycin, indicating the loss of chromosomal *aroE* and the absence of a plasmid copy. Three small transformants were isolated and the presence of the spectinomycin resistance gene in the *aroE* locus was verified by PCR (Figure S2). Genome sequencing using chromosomal DNA (cDNA) of the potential *aroE* mutants BP250, BP251 and BP254 and of the *E. coli aroA* mutant JW0891-1 (control) confirmed the absence of the *aroE* gene in selected small transformants (Figure 4A). No other sequence variation was identified other than the replacement of the EPSP synthase genes with the resistance markers.

To assess whether *aroE* can only be deleted on BHI plates, we transformed the wild type strain SP1 with cDNA of BP250 (*aroE::spc*) and propagated the bacteria on BHI and LB plates supplemented with spectinomycin. cDNA of the strain BP1303 (*gdpP::spc*) served as a control. As expected, many colonies emerged on the BHI plates, and as previously reported (Koo et al., 2017), very tiny transformants appeared on the LB plates (Figure 4B). Subsequent growth experiments with two isolated transformants that were obtained with the cDNAs of BP1303 (*gdpP::spc*) and BP250 (*aroE::spc*) on BHI plates revealed that an *aroE* is not culturable on LB plates (Figure 4C). To conclude, in *B. subtilis* the EPSP synthase is dispensable and indispensable for growth in BHI and LB medium, respectively.

The *B. subtilis* EPSP synthase mutant requires menaquinone for growth

Next, we compared the growth requirements of *B. subtilis aroE* and *E. coli aroA* mutants lacking EPSP synthase activity. In contrast to *B. subtilis*, the EPSP synthase is not essential for growth of *E. coli* on LB plates (Baba et al., 2006). To study the effect of BHI on the growth of the *B. subtilis aroE* mutant, we cultivated the strains SP1 (wild type) and BP250 (*aroE::spc*) in BHI, LB and in a 1:1 mixture of BHI and LB. As a comparison, we grew the *E. coli* wild type strain and the *aroA* mutant in LB, M9 minimal medium and in a 1:1 mixture of LB and M9. As shown in Figure 5A, the growth yield of the *B. subtilis* wild type strain was higher in BHI medium. Moreover, both, the growth rate and yield of the wild type was higher than that of the *aroE* mutant whose growth was dependent on BHI (Figure 3B). By contrast, the *E. coli* wild type and *aroA* mutant showed no growth difference in BHI and LB medium (Figure S3) but the growth of the *aroA* mutant was dependent on LB medium (Figure 5B). From this observation we conclude that the nutritional requirements of the EPSP synthase-deficient *B. subtilis* mutant appear to be more complex than those of *E. coli*.

Next, we aimed to identify the shikimate pathway-derived substance that would enhance the growth of the *B. subtilis aroE* mutant. Unlike *E. coli*, the transporters for the uptake of phenylalanine and tyrosine in *B. subtilis* are currently unknown (Figure 1). However, since the *aroE* mutant grows in BHI medium and the aromatic amino acids are essential building blocks for

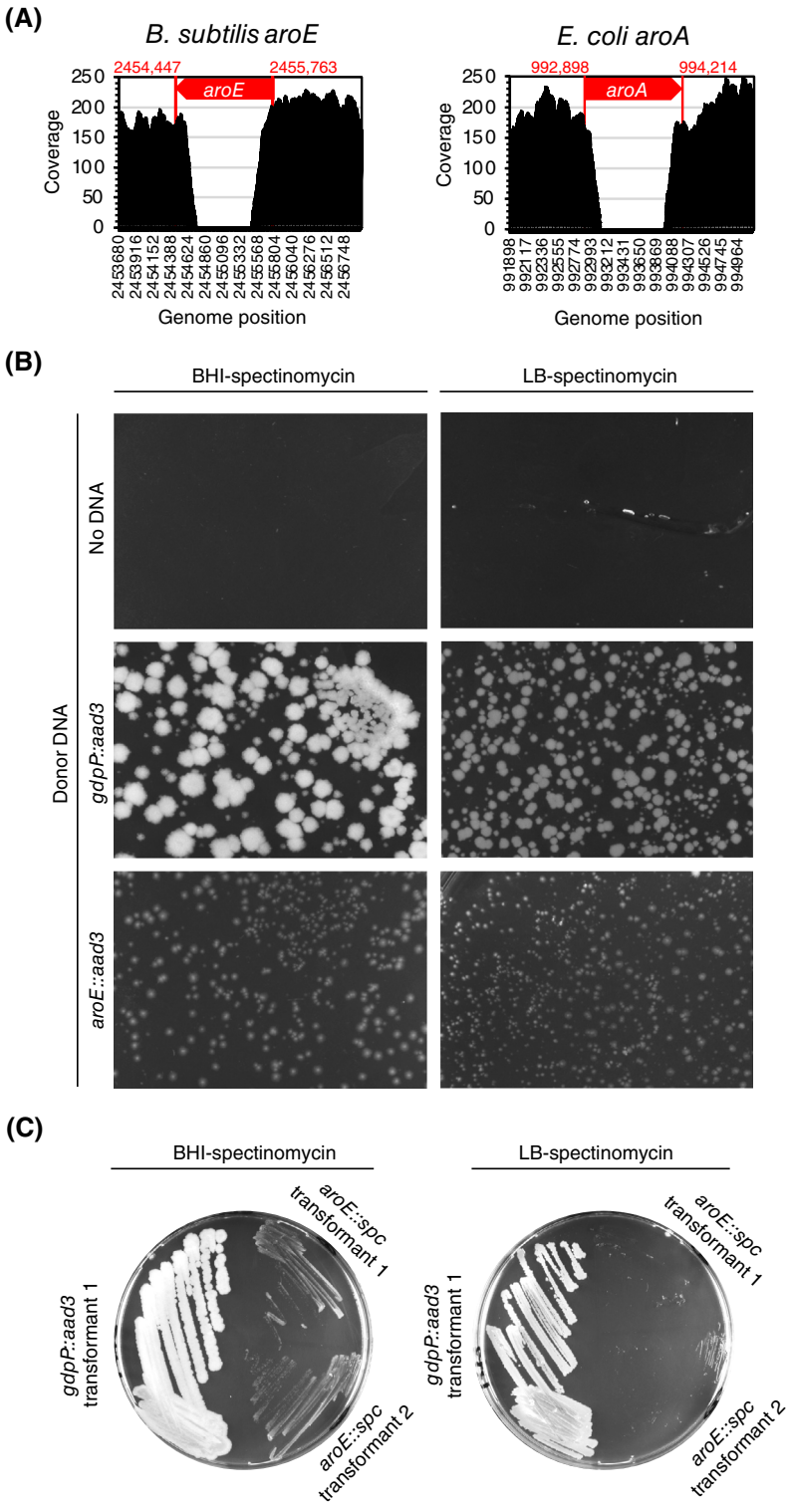

FIGURE 4 Inactivation of the *aroE* gene in *B. subtilis*. (A) Genome sequencing analysis to confirm the deletion of the *aroE* gene in *B. subtilis*. The genome of the *E. coli aroA* mutant JW0891-1 was sequenced as a control. The Illumina reads were mapped onto the *B. subtilis* and *E. coli* genomes CP058242 and CP009273, respectively, using the Geneious software package (Kearse et al., 2012). (B) Transformation experiment to assess the growth of the *B. subtilis aroE* mutant on BHI and LB medium. The chromosomal DNAs for the transformation of the *B. subtilis* strain SP1 was isolated from the strains BP250 (*aroE::spc*) and BP1303 (*gdpP::spc*). The bacteria were propagated on BHI and LB plates supplemented with spectinomycin and incubated for 24 h at 37°C. (C) Growth of transformants that were isolated from the BHI plates shown in B. on BHI and LB plates supplemented with spectinomycin. The plates were incubated for 24 h at 37°C.

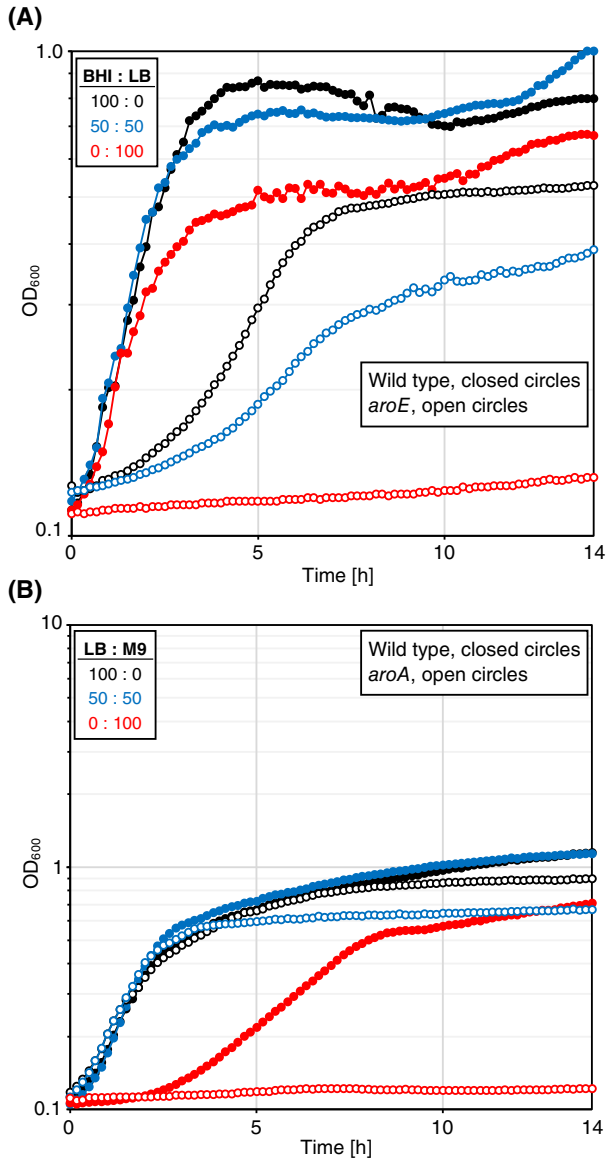

FIGURE 5 Dependency of the *B. subtilis* and *E. coli* EPSP synthase mutants on rich medium. The *B. subtilis aroE* mutant BP250 (A) and the *E. coli aroA* mutant JW0891-1 (B) were cultivated in BHI:LB and LB:M9 mixtures at 37°C. Each point in the growth curve is the mean of three measurement points (OD_{600}).

protein biosynthesis, we assumed that *B. subtilis* can take up all three aromatic amino acids. Furthermore, folate species including tetrahydrofolate are essential cofactors in the basic metabolism of *B. subtilis* and *E. coli* (Figure 1). Therefore, we also assumed that a metabolite downstream of the EPSP synthase, or the folate precursor and breakdown product *para*-aminobenzoate (pABA) and *para*-aminobenzoate-glutamate (pABA-Glu), respectively, must be taken up by both bacteria. Indeed, in *E. coli* it has been shown that the AbgT transporter enables growth of pABA auxotrophs with exogenous pABA-Glu (Hussein et al., 1998; Carter et al., 2007). Moreover, pABA can diffuse across biological membranes and rescues bacterial growth at nanomolar concentrations independent of *abgT* expression (Tran & Nichols, 1991; Maynard et al., 2018). We speculated that the growth of the *B. subtilis aroE* mutant in BHI and LB medium could be enhanced by quinone supplementation. For the growth experiments we used menaquinone 4 (MK-4) containing four isoprenoid side chain residues. As shown in Figure 6A,B, the *aroE* mutant grew almost like the wild type in BHI and LB medium supplemented with MK-4. From this we conclude that the quinone precursors or quinones present in the BHI medium enable but also limit the growth of the *B. subtilis aroE* mutant. Moreover, the *aroE* mutant cannot grow in LB medium unless MK-4 is provided by the environment.

Overexpression of the *B. subtilis aroE* gene increases GS resistance of *E. coli*

Based on the previous reports and this study, it remains unclear why *B. subtilis* and related *Bacilli* inactivate the *gltT* glutamate transporter gene and never accumulate mutations in *aroE* when exposed to GS (Figure 2A–D; Wicke et al., 2019). The finding that the *aroE* gene is only dispensable for growth of *B. subtilis* in rich medium suggests that the bacteria require an intact EPSP synthase under nutrient poor conditions. To compare the GS sensitivity of the EPSP synthases from *B. subtilis* and *E. coli*, we introduced the plasmids pBP143 (*aroA*) and pBP765 (*aroE*) into the *B. subtilis aroE* and *E. coli aroA* mutants BP250 and JW0891-1, respectively. As shown in Figure 7A,B, the overexpression of the *aroA* and *aroE* EPSP synthase genes complemented the *E. coli aroA* and *B. subtilis aroE* mutants. Moreover, the overexpression of the *B. subtilis aroE* gene strongly increased GS resistance of *E. coli* whose growth is completely inhibited with 10–15 mM GS (Wicke et al., 2019). By contrast, the *B. subtilis aroE* mutant synthesizing AroA from *E. coli* did not grow with 2.5 GS and the bacteria only tolerated low amounts of GS (5 mM) when the native EPSP synthase was overproduced (Figures 7 and S4). Thus, the EPSP synthases differ with respect to their GS sensitivity and probably also in their catalytic activity.

Genetic instability of the *E. coli aroA* C358A allele conferring GS resistance

Previously, we have observed that *E. coli* rapidly becomes GS resistant by acquiring mutations in the *aroA* gene or that affect its expression (Wicke et al., 2019). For instance, the amino acid substitution R120S allows *E. coli* to grow in the presence of 10 mM GS (Wicke et al., 2019). To further characterize the AroA R120S variant, we attempted to clone the *aroA* C358A allele using the shuttle vector pBQ200 (Martin-

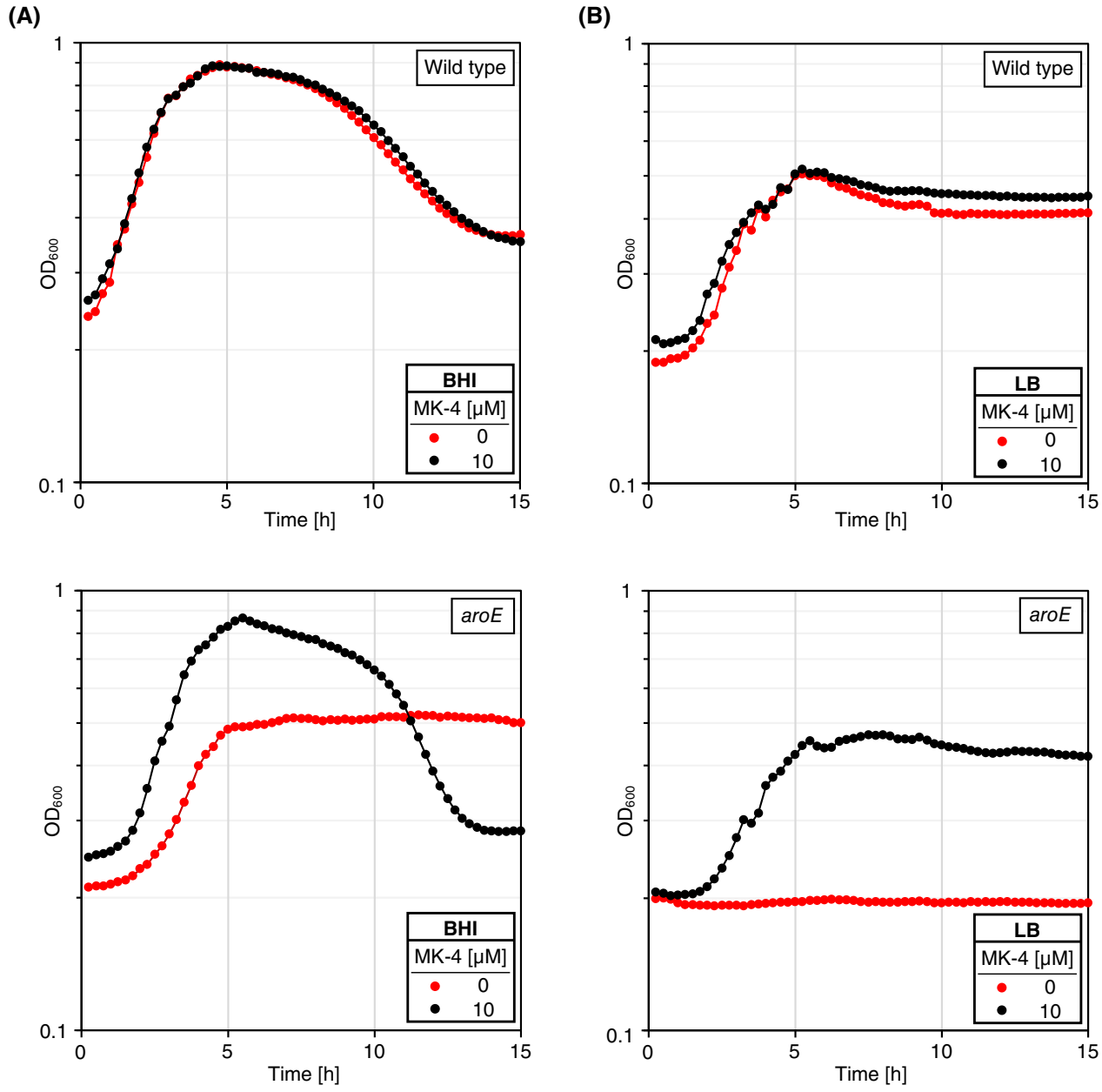

FIGURE 6 The *B. subtilis aroE* mutant requires MK-4 for growth in LB medium. Effect of MK-4 on the growth of the *B. subtilis* laboratory strain SP1 and the *aroE* mutant BP250 in BHI and LB medium at 37°C. Each point in the growth curve is the mean of three measurement points (OD_{600}).

Verstraete et al., 1994). For this purpose, the *aroA* C358A allele was amplified by colony PCR using cells of the GS-resistant *E. coli* strain BPE1 as a template. DNA sequencing analyses revealed that among 38 plasmids only one contained the *aroA* allele with the single C358A substitution (Table 2). In 37 plasmids, the *aroA* gene contained the C358A mutation and at least one additional mutation, which cause amino acid substitutions in the encoded EPSP synthase (Figure 8A–C). Furthermore, three plasmids contained *aroA* alleles with frameshift mutations that would truncate the AroA protein after 33, 240 and 394 amino acids (Figure 8B). Moreover, one plasmid encodes the AroA A427S variant, which is extended by a glutamate residue at its C terminus (Figure 8B). Next, we calculated the double changes in Gibbs free energy (ΔΔG), which is a metric for predicting how amino acid exchanges will affect the stability of a protein (Kellog et al., 2011; Park et al., 2016). The calculation of the ΔΔG for the AroA variants revealed that the R120S substitution and all other amino acid substitutions very likely would affect the stability of the EPSP synthases (Figure S5). Several *aroA* alleles in the constructed plasmids also contained synonymous mutations that

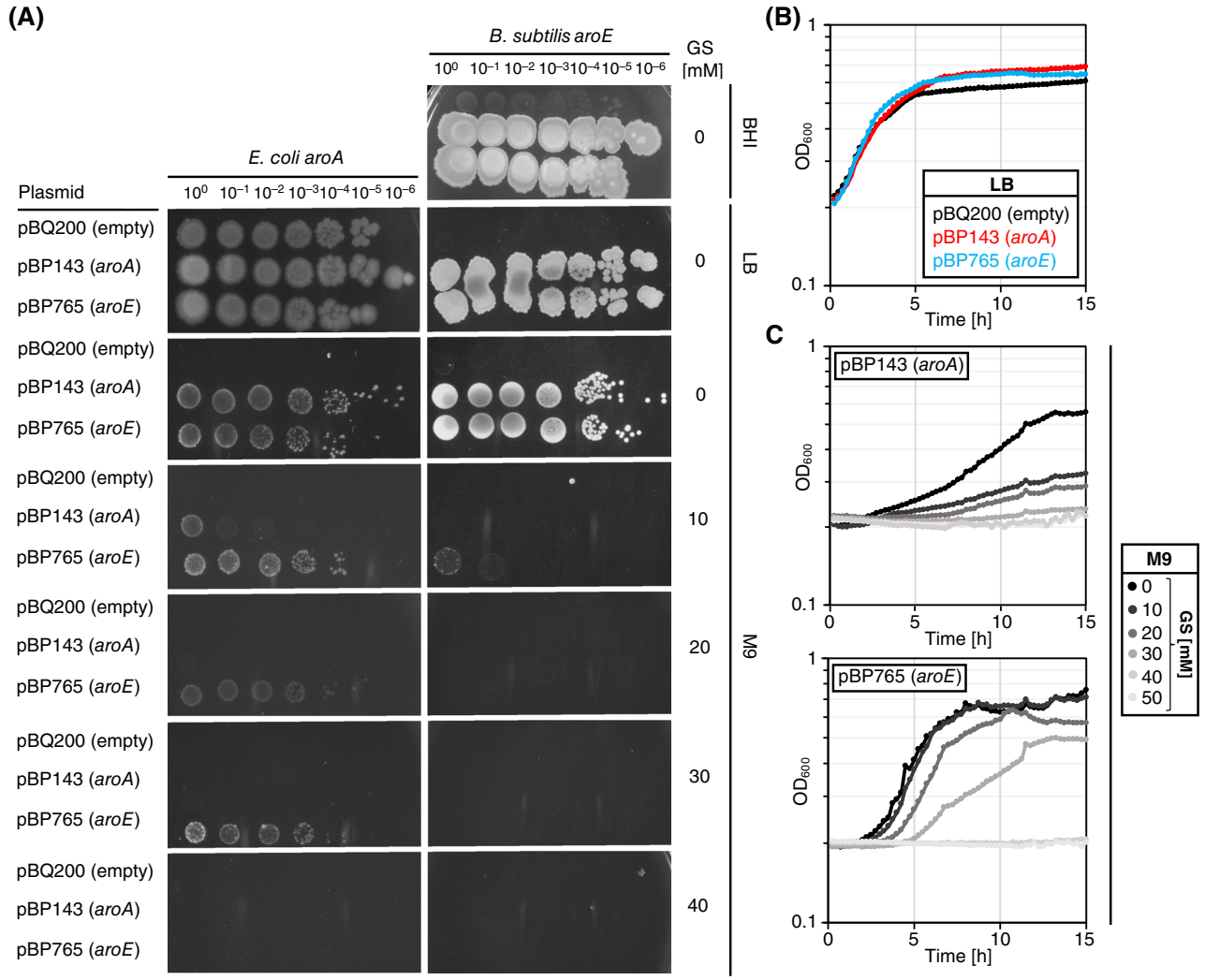

FIGURE 7 Overexpression of the *B. subtilis aroE* gene confers GS resistance in *E. coli.* (A) For the drop dilution assay, the *B. subtilis aroE* and the *E. coli aroA* mutants BP250 and JW0891-1, respectively, carrying the plasmids pBQ200 (empty), pBP143 (*E. coli aroA*), and pBP765 (*B. subtilis aroE*) were grown over night in BHI and LB medium at 28°C. The cells were washed 2× in M9 medium and the OD_{600} was adjusted to 1.0. A total of 5 μL of the indicated dilutions were spotted onto BHI, LB and M9 plates that were incubated for 24 h at 37°C. (B) Growth of the *E. coli aroA* mutant JW0891-1 carrying the plasmids pBQ200 (empty), pBP143 (*aroA*) and pBP765 (*aroE*) in LB medium at 37°C. (C) Effect of GS on the growth of the *E. coli aroA* mutant JW0891-1 carrying the plasmids pBP143 (*aroA*) and pBP765 (*aroE*) in M9 medium at 37°C. Each point in the growth curve is the mean of three measurement points (OD_{600}).

could affect mRNA folding and stability or translation of the AroA variants (Table 2). We also isolated the AroA G14V R120S D313G T381A M389T T409S variant in which the aspartate at the position 313 has been previously described to be critical for the enolpyruvyl transfer reaction after the formation of the tetrahedral adduct of the substrates (Eschenburg et al., 2003). Therefore, it is likely that the AroA variant with the 6 amino acid substitutions is inactive. Complementation experiments using the *E. coli aroA* mutant JW0891-1 revealed 13 of the 37 AroA variants including the C-terminally truncated and the AroA G14V R120S D313G T381A M389T T409S variants are catalytically inactive (Table 2). Moreover, we isolated two AroA variants (R120S L301P and R120S L133P) in which only one additional amino acid substitution results in the full inactivation of the EPSP synthase (Figure 8). To conclude, unlike *B. subtilis, E. coli* rapidly acquires GS resistance by GS target modification. However, the genetic instability of the *aroA* C358A allele in *E. coli* indicates that enhanced GS resistance may cause fitness costs under non-selective growth conditions.

DISCUSSION

In the present study, we show that like the domesticated *B. subtilis* laboratory 168 (Wicke et al., 2019) also the undomesticated *B. subtilis* strain NCIB 3610 as well as environmental *Bacillus* isolates acquire GS

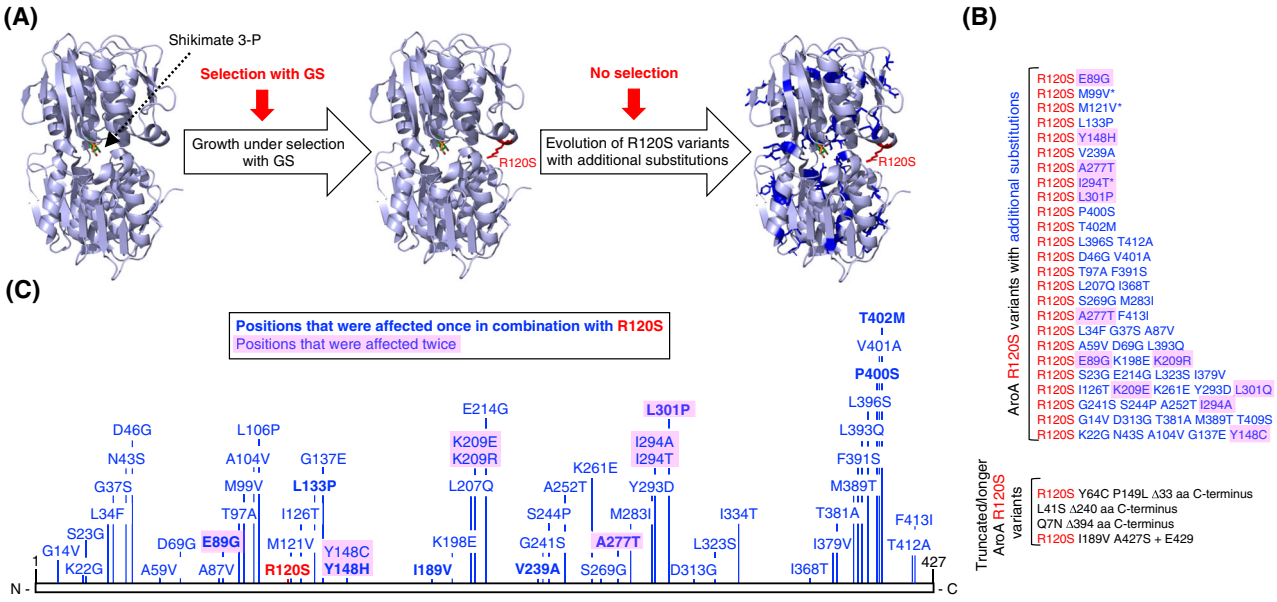

FIGURE 8 Evolution of the *E. coli* AroA R120S variant without selection pressure. (A) The GS-insensitive AroA R120S variant in the *E. coli* strain BPE1 was previously isolated during incubation of the *E. coli* laboratory strain W3110 on M9 plates supplemented with 10 mM GS (Wicke et al., 2019). Attempts to isolate and clone the *aroA* C358A allele from the *E. coli* strain BPE1 that was grown on LB medium under non-selective conditions (absence of GS) yielded in 37 constructed plasmids containing *aroA* alleles with additional mutations (Table 2). The AroA models were generated using the SWISS-Model server for homology modelling of protein structures (Waterhouse et al., 2018) and a model of the structure of the EPSP synthase from *E. coli* (PDBid: 1G6T) (Schönbrunn et al., 2001). (B) AroA variants with additional amino acid substitutions are shown in blue and truncated/longer AroA variants. Amino acid residues that were affected twice are highlighted in pink. The AroA variants labelled with an asterisk are encoded by *aroA* alleles that contain additional silent mutations (Table 2). (C) Distribution of additional substitutions over the AroA R120S primary sequence.

resistance through the mutational inactivation of the *gltT* glutamate transporter gene. Thus, this GS resistance mechanism does not appear to be inherent in domesticated laboratory strains of *B. subtilis*. Previously, we have shown that also a *B. subtilis* strain carrying two chromosomal *gltT* copies did not accumulate mutations in the *aroE* gene (Wicke et al., 2019). Even though the GS-resistant suppressor mutants appeared later and at a lower frequency, all characterized mutants had both *gltT* copies inactivated (Wicke et al., 2019). This observation suggests that the *B. subtilis* EPSP synthase does not tolerate amino acid exchanges that would reduce the catalytic activity of the enzyme, thereby causing a growth defect under the tested conditions. Indeed, in many biological systems, including *B. subtilis*, it has been observed that amino acid exchanges in the EPSP synthase simultaneously decrease GS sensitivity and the enzyme's catalytic efficiency (Majumder et al., 1995; Selvapandiyan et al., 1995; Sikorski & Gruys, 1997; Chekan et al., 2016; Han et al., 2017; Vila-Aiub et al., 2019). However, we also confirmed that AroE of *B. subtilis* belongs to the class II EPSP synthases that are less sensitive to GS (Figure 7A–C; Priestman et al., 2005; Leino et al., 2021). Structural studies uncovered that class II EPSP synthases, unlike GS-sensitive class I EPSP synthases, possess a cation binding site that reduces GS sensitivity of the enzymes (Light et al., 2016). Surprisingly, our growth experiments with *B. subtilis* and *E. coli* synthesizing either AroA or AroE revealed that the *B. subtilis* EPSP synthase only conferred high-level GS resistance in *E. coli*. It is tempting to speculate that the uptake of GS via the glutamate transporters is more efficient in *B. subtilis* than in *E. coli*. Previously, we have indeed observed that the *E. coli* glutamate transporter GltP did not fully restore GS sensitivity of a *B. subtilis gltT* mutant (Wicke et al., 2019). Thus, the fact that *B. subtilis* has fewer but possibly more efficient GS uptake systems could also explain why the bacteria primarily inactivate the *gltT* and *gltP* glutamate transporter genes during growth with GS. However, the question why *B. subtilis* always inactivates the glutamate transporter genes in the presence of GS has not yet been satisfactorily answered. A plausible answer to this question could be that more than one target exists in *B. subtilis*. To test this hypothesis, it is necessary to study the influence of GS on the growth of an *aroE* mutant in minimal medium (Wicke et al., 2019; Schwedt et al., 2023). Unfortunately, our attempts to cultivate the *aroE* mutant in minimal medium failed.

Recently, it has been shown that phylogenetically distantly related bacteria such as the Gram-negative β-proteobacterium *Burkholderia anthina* also do not

accumulate mutations in the EPSP synthase gene (Schwedt et al., 2023). In all isolated GS-resistant mutants, the intracellular PEP concentrations were increased due to the inactivation of the *ppsR* gene, encoding a regulator of the PEP synthetase PpsA (Schwedt et al., 2023). Very likely, the increased cellular PEP concentration hinders GS from inhibiting the EPSP synthase in the evolved *B. anthina* mutants. Thus, bacteria like *B. anthina* and *B. subtilis* are obviously dependent on a catalytically efficient EPSP synthase to supply the cell with sufficient precursors for the biosynthesis of building blocks and cofactors. Interestingly, the UDP-*N*-acetylglucosamine enolpyruvyl transferase MurA that, like the EPSP synthase, belongs to the small family of enolpyruvyl transferases also does not tolerate amino acid substitutions *in vivo* (Chekan et al., 2016; Dewachter et al., 2023). MurA is an essential enzyme that is inhibited by the epoxid antibiotic fosfomycin and catalyzes the first committed step in peptidoglycan biosynthesis in Gram-negative and Gram-positive bacteria (Kahan et al., 1974). Thus, MurA and to some extent also the EPSP synthase are highly attractive antibiotic targets (Frlan, 2022; Dewachter et al., 2023). However, in the case of *B. subtilis* and closely related species only antibiotics that are not taken up via GltT are suitable because the bacteria rapidly accumulate loss-of-function mutations in the *gltT* glutamate transporter gene.

Beside the mutational inactivation of GS uptake systems, two other GS resistance mechanisms have been described in bacteria. For instance, bacteria can accumulate mutations that either reduce the GS-binding affinity of the EPSP synthase or increase the cellular amount of the enzyme. Indeed, *E. coli* rapidly acquires mutations in the *aroA* gene that render the encoded enzyme less sensitive to GS (Wicke et al., 2019). Overproduction of the EPSP synthase to titrate GS away can be achieved in *E. coli* either by promoter-up mutations or by the amplification of a genomic region containing the *aroA* gene (Wicke et al., 2019). The latter GS resistance mechanism is also widespread in plants (Gaines et al., 2010; Dillon et al., 2017). It is also unclear why *B. subtilis* does not accumulate mutations in the genome that increase the cellular concentration of the EPSP synthase. It is tempting to speculate that the genomic context of the *aroE* gene in *B. subtilis* does not provide the genetic basis for promoting promoter-up mutations and gene amplification to increase the EPSP synthase levels. However, even though *E. coli* primarily modifies the target of GS, the synthesis of a GS-insensitive EPSP synthase like the R120S variant seem to be disadvantageous for the bacteria in the absence of GS. Indeed, we observed that the GS-resistant *E. coli* mutant synthesizing the AroA R120S variant acquired additional mutations in the *aroA* gene that either reduce the activity of the EPSP synthase or even fully inactivate the enzyme (Figure 8; Table 2). Thus, the metabolic flux through the shikimate pathway needs to be balanced, probably to prevent the accumulation of toxic intermediates.

The present study also confirms that the first four reactions of the shikimate pathway in *B. subtilis* can be bypassed by yet unknown promiscuous enzymes during growth in LB rich medium (Figure 1 and Table S6; Koo et al., 2017). Moreover, we found that the *B. subtilis* EPSP synthase mutant is only viable in BHI or in LB medium supplemented with MK-4 (Figures 5 and 6). As similar observation has been made previously with a *Listeria monocytogenes* EPSP synthase mutant whose growth defect could be relieved by quinones (Stritzker et al., 2004). Our attempts to isolate spontaneous *aroE* suppressor mutants that show improved growth on BHI plates and in liquid medium failed. This indicates that the EPSP synthase reaction in *B. subtilis* cannot be bypassed by recruiting another enzyme or a transporter that channels intermediates into the shikimate pathway. In contrast to *B. subtilis*, the *E. coli* EPSP synthase mutant could grow in LB medium without quinone supplementation. Moreover, it has even been reported that all seven reactions of the shikimate pathway are dispensable for growth of *E. coli* in LB (Figure 1; Baba et al., 2006). Thus, beside the known transport systems for the uptake of the aromatic amino acids, *E. coli* must also possess transporters for the uptake of the precursors for folate and quinone biosynthesis that are different in *B. subtilis*. In conclusion, the different equipment of *E. coli* with transport systems and the existence of promiscuous enzymes causing underground metabolism could explain the dispensability of the shikimate pathway enzymes in this organism. The higher demand of *B. subtilis* for shikimate pathway intermediates could also explain the low mutational flexibility of the *aroE* gene encoding an intrinsically GS-insensitive EPSP synthase.

In the future, it will be interesting to study the effect of GS on the growth of the *B. subtilis* and *E. coli* EPSP synthase mutants in minimal medium. This analysis will reveal for the first time whether GS inhibits other enzymes in the model bacteria in addition to the EPSP synthase.

Moreover, the transporters for the uptake of folate precursors and of the aromatic amino acids phenylalanine and tyrosine need to be identified and characterized in *B. subtilis*.

AUTHOR CONTRIBUTIONS

Fabian M. Commichau: Conceptualization; investigation; funding acquisition; writing – original draft; writing – review and editing; visualization; supervision; resources. **Inge Schwedt:** Conceptualization; investigation; methodology; supervision; resources; visualization. **Kerstin Schöne:** Investigation; methodology; supervision. **Maike Eckert:** Investigation. **Manon Pizzinato:** Investigation. **Laura Winkler:** Investigation.

Barbora Knotkova: Investigation. **Björn Richts:** Investigation; supervision. **Jann-Louis Hau:** Investigation. **Julia Steuber:** Supervision. **Raul Mireles:** Investigation. **Lianet Noda Garcia:** Supervision. **Günter Fritz:** Supervision. **Carolin Mittelstädt:** Supervision. **Robert Hertel:** Investigation; formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/datasets/genome

ORCID

Jann-Louis Hau https://orcid.org/0009-0002-7478-4234
Fabian M. Commichau https://orcid.org/0000-0003-0323-7479

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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