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Bacterial glyphosate resistance conferred by overexpression of an *E. coli* membrane efflux transporter

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Abstract Glyphosate herbicide-resistant crop plants, introduced commercially in 1994, now represent approximately 85% of the land area devoted to transgenic crops. Herbicide resistance in commercial glyphosate-resistant crops is due to expression of a variant form of a bacterial 5-enolpyruvylshikimate-3-phosphate synthase with a significantly decreased binding affinity for glyphosate at the target site of the enzyme. As a result of widespread and recurrent glyphosate use, often as the only herbicide used for weed management, increasing numbers of weedy species have evolved resistance to glyphosate. Weed resistance is most often due to changes in herbicide translocation patterns, presumed to be through the activity of an as yet unidentified membrane transporter in plants. To provide insight into glyphosate resistance mechanisms and identify a potential glyphosate transporter, we screened *Escherichia coli* genomic DNA for alternate sources of glyphosate resistance genes. Our search identified a single non-target gene that, when overexpressed in *E. coli* and *Pseudomonas*, confers high-level glyphosate resistance. The gene, *yhhS*, encodes a predicted membrane transporter of the major facilitator superfamily involved in drug efflux. We report here that an alternative mode of glyphosate resistance in *E. coli* is due to reduced accumulation of glyphosate in cells that overexpress this membrane transporter and discuss the implications for potential alternative resistance mechanisms in other organisms such as plants.

Keywords Glyphosate · Herbicide · Major facilitator superfamily · Membrane transporter

Introduction

Herbicide application is the predominant form of weed control in agriculture, with the broad-spectrum herbicide glyphosate being one of the most popular. The widespread introduction and success of glyphosate-resistant crops, such as Roundup-Ready corn, soybean, cotton, and oilseed rape, have contributed significantly to the increased use of glyphosate herbicide [25]. Currently, glyphosate-resistant crops represent approximately 85% of the land area devoted to transgenic crops; in 2009, more than 134 Mha of transgenic crops were planted [8].

Glyphosate kills plants through the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an essential enzyme in the production of aromatic amino acids and many other secondary products. Herbicide resistance in commercial glyphosate-resistant crops is due to expression of a variant form of the EPSPS which prevents binding of glyphosate to the enzyme [16, 29]. The widespread and recurrent use of herbicides for weed control has resulted in the selection of more than 323 resistant biotypes in 183 weedy species [3, 34], including 21 weedy species with resistance to glyphosate [15]. Interestingly, mutation in the herbicide binding site in EPSPS (target-site resistance) is rare, whereas non-target-site resistance has evolved and is likely the major cause for most resistant biotypes [6, 34].

Non-target-site herbicide resistance may arise through several mechanisms (reviewed in Yuan et al. [34]) and

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changes in translocation have been implicated in a few weedy species ([7, 10, 17, 21, 22, 32]). Numerous recent studies on horseweed (*Conyza canadensis* L.) have implicated changes in glyphosate translocation in resistant biotypes (reviewed in Preston and Wakelin [24], Shaner [30]). The view that emerges from these studies [14] suggests that glyphosate enters sensitive and resistant cells at the same rate, but is rapidly sequestered into vacuoles in resistant biotypes. Glyphosate that is sequestered in the vacuole is removed from the cytoplasmic pool available for translocation to sensitive meristematic sink tissues. On the other hand, sensitive biotypes succumb to the lethal effects of glyphosate because there is a greater reservoir of glyphosate in the cytoplasm of source and sink tissues [14]. Characterization of the transcriptome from sensitive and resistant horseweed has identified several differentially expressed transcripts, with transporter genes among them implicated as candidates for a putative non-target herbicide resistance gene [20, 33].

In *Escherichia coli*, amplification of the *aroA* EPSPS gene renders cells resistant to glyphosate [27]. The objectives of the current study were to further examine glyphosate resistance mechanisms in bacteria and determine if a non-target glyphosate resistance gene could be identified. Utilizing a bacterial screen, we identified a single non-target gene that when overexpressed in *E. coli* and *Pseudomonas* confers high-level glyphosate resistance. The gene, *yhhS*, encodes a highly conserved, predicted membrane transporter of the major facilitator superfamily involved in drug efflux. We report here that an alternative mode of glyphosate resistance in *E. coli* is due to reduced accumulation of glyphosate in cells that overexpress this membrane transporter and discuss the implications for potential alternative resistance mechanisms in other organisms such as plants.

Methods

Screening of an *E. coli* genomic DNA library and cloning of the *yhhS* gene

DNA from *E. coli* strain SR20 (GM42 *hfr, his-, dam3-*) was digested with *Mbo*I and run on a sucrose gradient. DNA fractions were collected and fragment size was monitored by agarose gel electrophoresis. Two genomic DNA fractions containing 10- to 15-kb-sized fragments were collected and concentrated by precipitation. DNA from each size fraction was ligated independently into the *Bam*H1 site of plasmid pBR327. Selection of *E. coli* transformants on glyphosate-containing M9 minimal media resulted in 22 colonies with strong growth against a background of pinpoint colonies. Single colonies were isolated from each glyphosate-resistant line and plasmid DNA was purified from all clones. Plasmid DNA was subsequently transformed back into strains SR400 (*aroA*−) and SR417 (*aroA*+) to test the ability to grow in the absence of exogenously added aromatic amino acids, indicative of *aroA*+ activity.

Sequencing of the insert from the longest (approximately 3.5 kb) glyphosate-resistant *aroA*− clone (II-14) revealed an internal open reading frame with 100% identity to a portion of the *E. coli yhhS* gene, along with approximately 1 kb of upstream promoter sequence. PCR was then utilized to clone the truncated *yhhS* coding region found in the II-14 clone along with the full-length *yhhS* coding region. PCR products were cloned into pGEM-T for verification by subsequent DNA sequencing. The verified truncated (pMON49013) and full-length (pMON49014) coding region were then transferred into pET11 for inducible expression by the T7 RNA polymerase.

Screening media and growth conditions

For screening of glyphosate tolerance, *E. coli* strain SR20 was grown in M9 minimal media and strains SR400 (CGSC #AB1321) and SR417 (SR400 *aroA*+ by Pi phage transduction according to methods in Rogers et al. [27]) were grown at 37°C in VB minimal medium [31]. Media additions included 0.4% (w/v) glucose as carbon source, 100 µg/ml vitamin B1, histidine (100 µg/ml), ampicillin (50 µg/ml), and 10 mM glyphosate. BL21 DE3 and BL21 DE3 pLysS (Novagen) were grown in M9 media. Solid media plates were made by the addition of 1.5% Bacto agar.

Conjugation for transfer of *yhhS* into *Pseudomonas*

For scoring of glyphosate resistance in *Pseudomonas*, an approximately 2.0-kb DNA fragment carrying the truncated *yhhS* coding sequence was cloned as a *Hin*dIII fragment into the *Hin*dIII site of a broad-host-range shuttle vector (pMON7340 [2]) to create pMON1590. Insertion of the *yhhS* coding sequence disrupts the kanamycin resistance *nptII* gene in pMON1590.

Transconjugation by triparental mating was used to transfer pMON1590 into *Pseudomonas aureofaciens* 3732 (*Rif*^{r} *Nal*^{r}) (Obukowicz et al., 1986) following the protocol of Barry [2]. Complementation functions were provided by pRK2013 (Ditta et al., 1980) in *E. coli* strain HB101. *Pseudomonas* transconjugates containing the *yhhS* gene were selected on L broth supplemented with 200 µg/ml ampicillin, 50 µg/ml rifampicin, and 10 µg/ml tetracycline. Single colonies were purified on the same medium for subsequent glyphosate growth assays.

Glyphosate accumulation assay

Cells were grown at 37°C in 250-ml flasks containing 50 ml L broth (containing 100 ppm ampicillin for plasmid-bearing strains). Inoculation used 3 ml from overnight-grown cultures. After 2 h of growth, 1 mM isopropyl-β-D-thiogalactoside (IPTG) was added. Cells were harvested after an additional 2 h of growth, centrifuged at 7,000 rpm in 50-ml centrifuge tubes for 10 min, and resuspended in a final volume of 6 ml of L broth (plus 100 ppm ampicillin where relevant). The cell suspension was divided between two 10-ml culture tubes and 100 µl of ^{14}C-glyphosate in 10 mM unlabeled glyphosate was added to each tube. Cells were then allowed to grow for an additional 3 h at 37°C. Cells were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS) (5 ml each wash), and ^{14}C-radiolabel in harvested cells, media, and washes was quantified.

Bioinformatic analysis

The homologous protein sequences to yhhS were identified by position-specific iterated blast of the *Enterobacteriaceae* yhhS amino acid sequence against the UNIREF90 protein sequence database (EMBL-EBI). Approximately 120 bacterial sequences were identified with e-values less than 1×10^{-30} and hit and query coverage greater than 80%. The phylogenetic tree of these sequences is shown in Supplemental Fig. 1. The multiple sequence alignment is made using Muscle3.7 [9] and the figures were created using ICMpro software [1].

Results

Screening *E. coli* for glyphosate resistance genes

A plasmid DNA library of 10- to 15-kb size-selected genomic DNA fragments from *E. coli* strain SR20 (*aroA*+) was created and transformed back into *E. coli*. Selection on M9 media plates containing 10 mM glyphosate identified 22 resistant clones.

Since an *aroA*+ strain was used as the source of genomic DNA, further screening was necessary to distinguish glyphosate resistance conferred by the *E. coli aroA* gene, encoding EPSPS, from resistance caused by other mechanisms. Plasmid DNA from all 22 clones was purified and retransformed into strains SR400 (*aroA*−) and SR417 (*aroA*+). Growth on glyphosate-containing minimal media in the absence of exogenously added aromatic amino acids when grown in the SR400 background suggested recovery of the *aroA* gene from the original selection scheme. Five of the original clones were able to complement the *aroA*− phenotype and were not tested further. Growth on glyphosate-containing media of the remaining 17 isolates only in the SR417 strain background suggested the original selection scheme had identified an alternative mode of glyphosate resistance in those clones.

The alternative glyphosate-resistant clones were further characterized by restriction enzyme digestion. The longest clones carried an approximately 3.5-kb DNA fragment, and nearly all clones carried an identical internal 2.8-kb *BstE*II DNA fragment (data not shown). These data indicated that all glyphosate-resistant clones identified in the original selection scheme derived from the same *E. coli* genomic locus.

Characterization of the alternative glyphosate resistance gene

Sequencing of the longest glyphosate-resistant *aroA*− clone revealed an internal open reading frame with identity to a portion of the *yhhS* gene, located at 77.78 min on the *E. coli* chromosome. Search of the public databases indicates that the *yhhS* gene is highly conserved among all bacterial species with available genome sequence information. Figure 1a shows a phylogenetic tree for the yhhS homologues from gamma-proteobacter species, and an extended tree (Supplemental Fig. 1) indicates homologues throughout the bacterial kingdom. The level of amino acid sequence identity within the gamma-proteobacter clade ranges from approximately 45 to nearly 100%, with the *Pseudomonas aeruginosa* homologue being the most divergent protein in the clade.

The cloned truncated gene contained the *yhhS* promoter region and an apparent open reading frame of 271 amino acids encoding a putative 28-kDa peptide, whereas the predicted full-length *yhhS* gene in *E. coli* encodes a 419 amino acid peptide of predicted molecular weight 43.78 kDa (Fig. 1b). Alignment of several bacterial sequences indicates that a frame shift mutation that created a premature stop codon was introduced in the cloned portion of the truncated *yhhS* gene during the library screen. The full-length *yhhS* gene is annotated as a plasma membrane protein with 12 membrane-spanning domains, and is a member of the major facilitator superfamily (MFS) of membrane transporters. Interestingly, the truncated *yhhS* carries only seven of the predicted membrane-spanning domains (Fig. 1b), indicating that this is sufficient to confer glyphosate resistance when overexpressed.

The full-length membrane transporter enhances glyphosate resistance levels

Initial efforts to clone the full-length *yhhS* gene were unsuccessful, possibly due to lethality of the encoded full-length

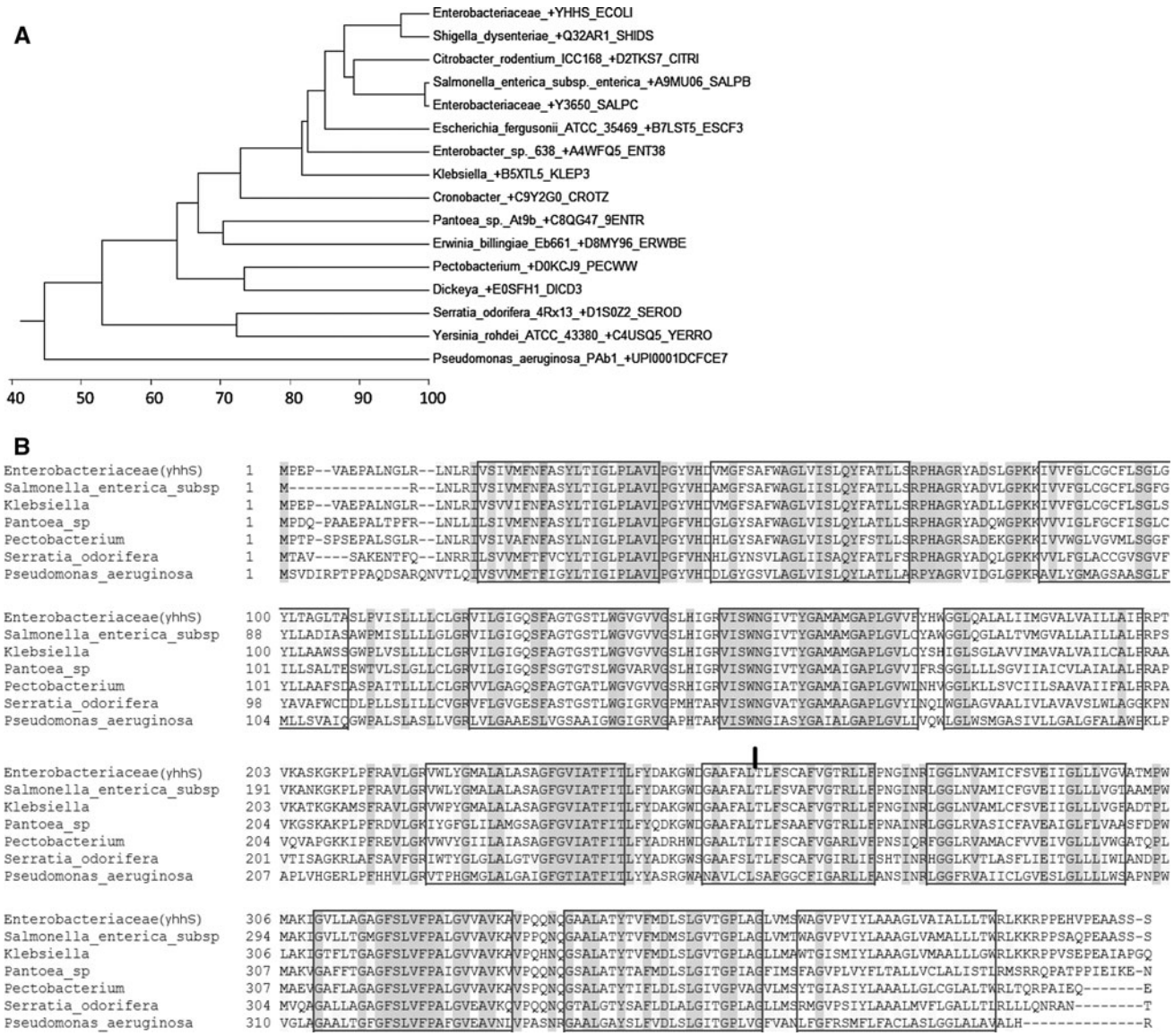

Fig. 1 Taxonomic tree and alignment of bacterial yhhS orthologues based on amino acid sequence. **a** The taxonomic tree shows the evolutionary distance (*horizontal axis*) based on yhhS sequences among all gamma-proteobacter species in the database. **b** Amino acid alignment of representative gamma-proteobacter species with the 12 predicted transmembrane-spanning domains *boxed*. Conserved amino acids are *shaded*. Note the position (*hash mark*) of the truncated open reading frame identified by sequencing in the originally selected glyphosate-resistant clone

membrane protein when constitutively overexpressed in *E. coli*. This result may explain why only a truncated version of the gene was recovered during our selection for glyphosate resistance. To circumvent potential lethality due to constitutive overexpression, and to compare glyphosate resistance encoded by the truncated and full-length *yhhS* coding regions, both were cloned into a pET vector such that expression of *yhhS* genes is dependent on an inducible host-encoded T7 RNA polymerase gene. *E. coli* hosts BL21 DE3 and BL21 DE3 pLysS were utilized to stringently control expression of the *yhhS* genes.

As seen in Fig. 2, using the inducible expression system, both the truncated (pMON49013) and full-length (pMON49014) *yhhS* genes allowed growth in both *E. coli* BL21 DE3 and BL21 DE3 pLysS strains in the presence of 15 mM glyphosate. These results suggest that accumulation of the full-length membrane protein is not lethal to *E. coli* if expression is stringently controlled. Further, multiple agar plate-based assays suggested that the full-length *yhhS* gene conferred enhanced growth on glyphosate-containing media when compared to the strains carrying the truncated gene (data not shown). Growth in liquid media (Fig. 2) also suggested that the full-length *yhhS* gene may allow for enhanced growth compared to the truncated *yhhS* gene in the presence of glyphosate. However, enhanced growth from the full-length *yhhS* gene was

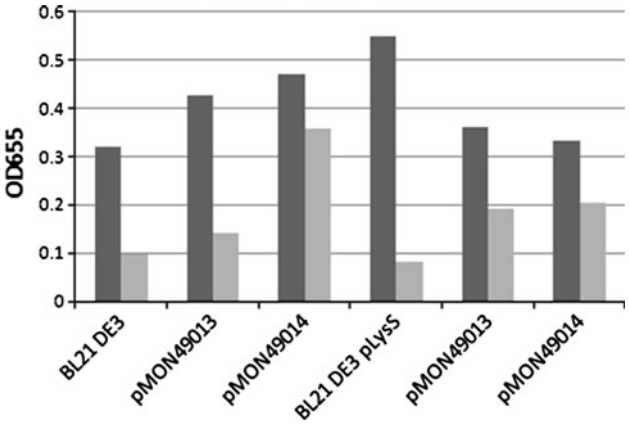

Fig. 2 Growth of bacterial strains in the presence or absence of the alternative glyphosate resistance gene. Growth of the BL21 DE3 or BL21 DE3 pLysS bacterial strains alone or carrying the truncated (pMON49013) or full-length (pMON49014) open reading frame of the glyphosate resistance gene. Bacteria were grown overnight in minimal media in the absence (*black bars*) or presence of glyphosate (10 mM, *gray bars*). IPTG (1 mM) was added and turbidity of cultures measured at OD655 after 24 h further growth in minimal media

only observed in the BL21 DE3 strain and both *yhhS* genes allowed growth to similar levels when in the BL21 DE3 pLysS strain (Fig. 2). Enhanced growth from the full-length yhhS while in the BL21 DE3 strain may be due to tighter control of induced expression and membrane protein accumulation in that strain, whereas the apparent lack of differential glyphosate resistance between the full-length and truncated *yhhS* constructs in the BL21 DE3 pLysS background may be due to leaky expression in that host that is detrimental to growth when the full-length protein is produced.

Glyphosate does not accumulate in cells that overexpress the yhhS transporter

To determine if glyphosate accumulates in cells, the fate of radiolabeled glyphosate added to growth medium was examined. Only the full-length *yhhS* gene (pMON49014) in the BL21 DE3 background was used for these studies. For these studies, bacteria were grown to high density in rich media, and glyphosate was added to the cultures 2 h after IPTG induction. Cells were harvested 3 h later, and radiolabeled glyphosate in both the cells and the media was quantified.

As can be seen in Fig. 3, at the lowest level (50 µl) of added radiolabeled glyphosate in the media, BL21 DE3 cells accumulate more than twice as much glyphosate as the strain carrying the pMON49014 construct. Furthermore, as the amount of exogenously added radiolabeled glyphosate in the media is increased, the accumulation of glyphosate in BL21 DE3 cells also increases dramatically. In contrast, cells that express the full-length yhhS membrane transporter from the pMON49014 construct do not significantly accumulate intracellular radiolabeled glyphosate at any concentration. These results suggest that glyphosate accumulation into *E. coli* is prevented by overexpression of the membrane transporter. However, we can not distinguish between lack of glyphosate uptake and active removal of glyphosate from cells during the 3-h incubation period.

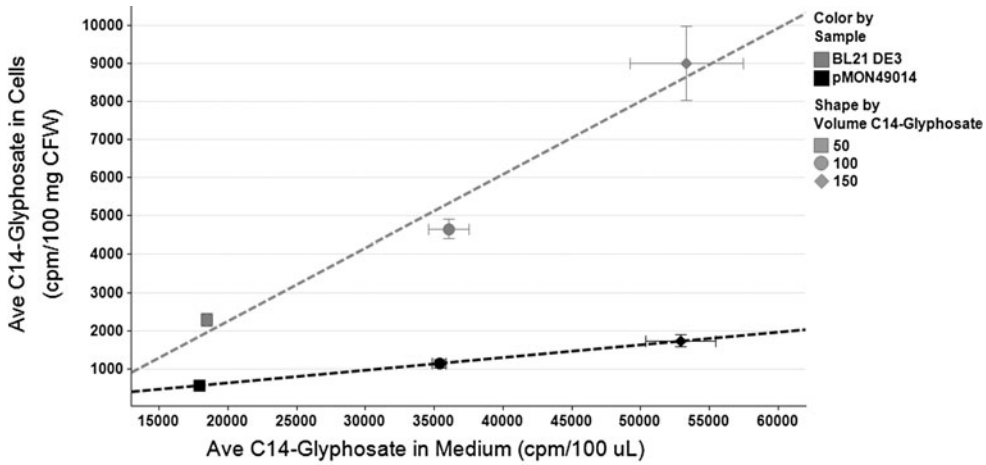

Fig. 3 Accumulation of radiolabeled glyphosate in bacterial cells or culture medium. BL21 DE3 cells (*gray*) or those carrying the full-length glyphosate resistance gene (pMON49014, black) accumulate ^{14}C-labeled glyphosate in the medium to similar amounts. The BL21 DE3 strain accumulates increasing levels of the ^{14}C-glyphosate in cells depending on added glyphosate amounts (50, 100, 150 µl volume), whereas pMON49014-containing cells accumulate very little glyphosate. Average (Ave) glyphosate in cells is normalized by fresh weight (CFW) of cultures after growth at each glyphosate concentration. Average (Ave) glyphosate in medium is normalized by volume. *Error bars* represent one standard deviation around the mean of two replicates for ^{14}C-glyphosate in cells (*vertical bars*) or in medium (*horizontal bars*). Note that *error bars* are not visible in some cases

The *E. coli* alternative glyphosate resistance gene confers glyphosate tolerance to *Pseudomonas*

To test the ability of the *E. coli* yhhS membrane transporter to confer glyphosate resistance to other bacterial species, we chose to use the species of gamma-proteobacter with the most divergent yhhS, *Pseudomonas aeruginosa*. The truncated *yhhS* coding region was used for this study, to avoid any complications due to potential lethality from overexpression of the full-length protein. We used a triparental mating scheme [2] to transfer the truncated *yhhS* coding region into *Pseudomonas* (see "Methods").

Pseudomonas transconjugates were purified by single colony isolation and their growth in the presence or absence of glyphosate was compared to similarly treated controls. Control lines included the *Pseudomonas* strain alone or carrying the helper plasmid pMON7340 without an inserted *yhhS* gene. Cultures were grown in liquid M9 media in the absence or presence of glyphosate and growth was monitored over 48 h at 30°C. Whereas all *Pseudomonas* lines grew to saturation by 24 h in the absence of glyphosate in the medium, only the transconjugate lines showed any growth on either 10 or 30 mM glyphosate-containing media (data not shown). No growth of the control cultures was observed even after 48 h. These data indicate that the truncated *E. coli* yhhS indeed can confer glyphosate resistance in *Pseudomonas*.

Discussion

We report here the discovery of a novel mechanism of bacterial glyphosate resistance from overexpression of a putative MFS transporter encoded by the *E. coli yhhS* gene. MFS transporters are one of the largest characterized families of bacterial multidrug transporters, which also include the ATP binding cassette (ABC), multidrug and toxic compound extruders (MATE), small multidrug resistance (SMR), and resistance-nodulation-division proteins (RND) (reviewed in Fluman and Bibi [11], Conte and Lloyd [5]). The MFS transporter superfamily can be further divided into at least 29 subfamilies (reviewed in Pao [19], Saier [28]), with at least 70 members in *E. coli* and 128 members predicted in the sequenced Arabidopsis genome ([26]; also see http://www.membranetransport.org).

Interestingly, MFS transporters are multi-specific, each capable of extrusion from bacterial cells of a variety of structurally dissimilar substrates (reviewed in Fluman and Bibi [11]). However, the natural substrates of these transporters are mostly unknown and drug resistance is manifested only after amplification of the transporter by overexpression. The results presented here are consistent with this mechanism, in that glyphosate resistance in *E. coli* and *Pseudomonas* was only conferred upon expression of *yhhS* from multicopy plasmids.

The yhhS transporter is predicted to contain 12 transmembrane-spanning domains, similar to many other members of the MFS family of transporters. From protein structural information, these proteins are expected to be organized into two pseudo-symmetrical six transmembrane domain bundles, which are packed against each other in the membrane (reviewed in Fluman and Bibi [11]). Interestingly, the original glyphosate resistance screen identified a truncated yhhS protein carrying only seven full-length putative membrane-spanning domains. Although the truncated protein is clearly able to transport glyphosate, expression of the full-length yhhS transporter apparently provided higher level glyphosate resistance in *E. coli*, suggesting that the structure of the truncated protein may not have been optimal.

The natural substrate for the yhhS transporter is unknown and we have not attempted to define its substrate specificity in detail. Although the exogenous substrates for numerous *E. coli* drug transporters have been investigated [18], *yhhS* was not included in those studies. Glyphosate is not naturally occurring but its use is widespread in agriculture. Thus, selection of glyphosate-resistant soil bacteria in fields that have been recurrently sprayed with glyphosate herbicide was the strategy used to identify the *EPSPS* transgene used in current commercially available Roundup-Ready herbicide-resistant crops. To our knowledge, overexpression of a membrane transporter in plants to provide herbicide resistance has not been attempted, though heterologous expression of an *Arabidopsis* MFS transporter, TPOI, does confer resistance to the herbicide 2,4-D in yeast [4].

Evolved herbicide resistance in weedy species has become more widespread in recent years [8]. The mechanisms of glyphosate resistance in weeds includes amplification of an *EPSPS* gene [12, 13, 23] and from alterations in glyphosate translocation patterns (reviewed in [3]). Currently, there is an effort to identify the transporter(s) responsible for altered translocation of glyphosate in resistant weeds, and the presence of a resistance-bearing ABC transporter has been implicated [20]. ABC transporters are also a large superfamily in bacteria and plants, with 112 predicted members in *Arabidopsis* (http://www.membranetransport.org). The results shown here suggest that the search for a plant glyphosate transporter should be expanded to include potential MFS transporter homologues. Furthermore, it may be possible to engineer herbicide resistance in transgenic plants by overexpression of a bacterial transporter, for example, using the yhhS transporter reported here to confer glyphosate resistance.

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