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Research Letter

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Profiling antibiotic resistance and electrotransformation potential of *Ensifer adhaerens* OV14; a non-*Agrobacterium* species capable of efficient rates of plant transformation

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One sentence summary: Profiling the antibiotic resistance of *Ensifer adhaerens* OV14, a non-*Agrobacterium* bacterium with the ability to genetically engineer plant genomes.

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ABSTRACT

Ensifer adhaerens OV14 underpins the successful crop transformation protocol termed *Ensifer*-mediated transformation but issues exist in regard to addressing the pleomorphic tendency of the bacterium in suboptimal conditions, identifying the optimal parameters for electrotransformation and defining the strain's antibiotic profile. Here, modifications made to growth medium composition addressed the pleomorphic trait of *E. adhaerens* OV14, delivering uniform *E. adhaerens* OV14 growth to ensure efficient rates of electroporation with plasmids up to 42.2 kb in size. Separately, 63 putative antibiotic resistance coding sequences were identified across the *E. adhaerens* OV14 genome, with testing confirming the strain's susceptibility to gentamicin (≥10 mg L^{-1}), tetracycline (≥10 mg L^{-1}), chloramphenicol (≥100 mg L^{-1}) and cefotaxime (≥500 mg L^{-1}) and resistance to ampicillin, paramomycin, streptomycin, spectinomycin, ticarcillin–clavulanate and kanamycin. Partial resistance against carbenicillin, rifampicin, hygromycin-B and neomycin was also recorded. Resistance to kanamycin was supported by seven independent nptII-like homologs located within the *E. adhaerens* OV14 genome. Transcriptional analysis of these targets highlighted two homologs (*AHK42288* and *AHK42618*) whose transcription was significantly elevated within 2 h exposure to kanamycin and which in the case of *AHK42288* was maintained out to 6 h post-exposure. In conclusion, our results have identified optimal conditions for the handling of *E. adhaerens* and have identified a future genome editing target (*AHK42288*) to negate the kanamycin-resistant phenotype of *E. adhaerens*.

Keywords: *Ensifer adhaerens* OV14; antibiotic resistance; plasmid; transformation; electroporation

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INTRODUCTION

Agrobacterium tumefaciens is a bacterial pathogen that causes 'crown gall' disease across a broad range of dicotyledonous and monocotyledonous species (Pitzschke and Hirt 2010). This occurs due to the pathogen's ability to transfer a particular DNA segment (T-DNA) of its tumor-inducing (Ti) plasmid into the nucleus of infected plant cells where it is then stably integrated and transcribed. Based around the technology platform *Agrobacterium*-mediated transformation (AMT), the exceptional ability of the bacterial pathogen to genetically transform plant cells in this manner underpins the current global market in engineered crop varieties.

While AMT is widely adopted as the system of choice for engineering novel varieties freedom to operate issues remain (Chi-Ham *et al*. 2012) especially in regard to regional-specific licensing (e.g. in the USA due to US Patent No. 8273954). In addition, the cost of securing regulatory approval for AMT-derived lines is significant and compounded by the plant pathogenic nature of *A. tumefaciens,* which in turn ensures that bringing engineered crop lines through commercialization is typically an option exclusive to the largest ag-biotech companies. Ideally, beginning to rectify this would require a gene transfer system that is dependent on a non-pathogenic organism with rates of transformation equivalent to that of AMT for both dicot and monocot species.

On this point, efforts have previously been made to detect alternative bacterial species with similar attributes to *A. tumefaciens* but strains identified have been limited in their efficacy (van Veen *et al*. 1988, 1989; Broothaerts *et al*. 2005). In contrast, we recently demonstrated that when equipped with the pCAMBIA5105 unitary plant transformation vector, the non-pathogenic *Ensifer adhaerens* OV14 is able to effectively transform the plant species *Solanum tuberosum*, *Nicotiana tobacum* and *Arabidopsis thaliana*, at rates analogous to the classic crop genome engineer, *A. tumefaciens*, in a process termed *Ensifer*-mediated transformation (EMT) (Wendt, Doohan and Mullins 2012).

By comparing the genome sequence of *E. adhaerens* OV14 against *A. tumefaciens* C58 and *Sinorhizobium meliloti* 1021 (a rhizobia with a propensity for low rates of genetic engineering; Broothaerts *et al*. 2005), it is clear that both *E. adhaerens* OV14 and *S. meliloti* 1021 possess homologs to all chromosomal-based genes cited as essential for *A. tumefaciens* induced genetic transformation. On the other hand, genes that exert a positive influence on the ability to transform a plant genome were present in the genome of *E. adhaerens* OV14 but absent from *S. meliloti* 1021 (Rudder *et al*. 2014).

However, to be able to exploit the full potential of *E. adhaerens* OV14 further investigation is required into the optimal environment required to manipulate and grow the strain. For instance, one issue with the use of *E. adhaerens* OV14 is its propensity to form aggregates in suboptimal conditions which complicates downstream EMT protocols such as accurately quantifying the optical density of the starter bacterial culture before treating the target plant tissues. With the exception of the original report by Casida (1982), which identified *E. adhaerens* as a non-obligatory bacterial predator, and supportive data from its phylogenetically related species, *S. morlense* (Wang *et al*. 2002), minimal knowledge exists on the antibiotic resistance and transformability of *E. adhaerens*. As plant transformation vectors have been designed to employ antibiotic selection markers (e.g. there is a heavy reliance on the use of kanamycin resistance) for selection in the transforming bacterium, determining the antibiotic profile of *E. adhaerens* OV14 is essential as is establishing the propensity of *E. adhaerens* OV14 to receive such plant transformation vectors.

The focus of this study was first to determine the optimal conditions required for the growth of *E. adhaerens* OV14 and its propensity to accept naked plasmids via electroporation. This was then complemented by *in silico* and *in vitro* assessments from which a comprehensive antibiotic profile for *E. adhaerens* OV14 was identified, which included a strong kanamycin-resistant phenotype. Data generated from the completion of both tasks will be of particular significance to users of strain OV14 and the broader species of *E. adhaerens*. More specifically, the additional characterization in this study of putative kanamycin-resistant genes provides potential targets for CRISPR/Cas9 genome editing, which has been described as a realistic protocol with which to edit prokaryotic genomes (Jiang *et al*. 2013).

MATERIALS AND METHODS

Evaluating optimal media and growth parameters

To identify a medium which minimized aggregate formation, temporal growth patterns of *E. adhaerens* strain OV14 were evaluated in Luria–Bertani (LB) medium, Tryptone–Yeast extract (TY), Yeast–mannitol (YM) broth, CASO broth (Fluka, 22098), Yeast extract peptone (YEP) media, Nutrient broth (NB) (Fluka, 03856), Teagasc Tryptone Yeast extract (TTY) media and Luria-Bertani (LBS; Sigma, L3022) (see Supplementary data). Other growth conditions tested were pH (5.5–7.5), temperature ranges (26–29°C) and revolutions per minute (200–240 rpm). Optical density at 600 nanometer wavelength (OD_{600nm} values) was recorded every 24 h for at least 96 h post-inoculation using a plate reader (Biotek Synergy HT, USA). Starter cultures for examining growth conditions were initiated using a 10 μL aliquot of a −80°C stock culture in 20 mL of TTY media.

Electroporation potential of *E. adhaerens* strain OV14

The capacity of *E. adhaerens* OV14 to receive plasmids of varying sizes (Table 1) was assessed using a modified protocol of previously published methods (Dower, Miller and Ragsdale 1988; Wen-jun and Forde 1989) with the factors of media for electrocompetent cell preparation (LB and TTY), recovery of cells post pulse (SOC and TTY), and time of recovery (1 and 4 h) examined systematically (see Supplementary data). For electroporation, cells were thawed on ice before being transferred to a prechilled 0.2 cm electroporation cuvette (Geneflow, E6–0060). Plasmid DNA (200 ng) was added and the mix incubated for 30 min on ice. Pulses were applied from a Bio-Rad Gene Pulser (25 μF capacitance, resistance of 400 Ω and 2.5 kV/cm voltage) giving a time constant of 10 ms. Cells were recovered by adding 1 mL of TTY to the cuvette, which was then transferred to a final volume of 10 mL TTY broth, with cells then incubated for 4 h at 28°C, 220 rpm. Aliquots of 50 μL were plated on TTY agar containing appropriate antibiotics and incubated at 28°C for 72 h. Colony appearance was recorded every 24 h after plating. Transformation efficiencies were calculated as transformants per μg^{-1} of DNA used for electroporation. Single colonies were picked to inoculate 20 mL TTY broth (plus respective antibiotic selection) for PCR-based confirmation (see Table S1, Supporting Information, for details).

In silico and *in vitro* investigation of antibiotic resistance

Annotated genes in the *E. adhaerens* OV14 genome conferring antibiotic resistance were recovered from the KEGG and NCBI Protein databases (http://www.genome.jp/kegg/;

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Table 1. List of plasmids, their relative selection markers, size and type of plasmid used in this study for electroporation of *E. adhaerens* OV14.

Plasmid	Relevant markers[a]	Size of plasmid (bp)	Vector type	Source of reference
pRU1144	Gent^r	5311	Bacterial expression	Karunakaran *et al.* (2005)
pRU1161	Tet^r	13079	Bacterial expression	Karunakaran *et al.* (2005)
pC2201	Chlor^r	11785	Binary vector for plant transformation	Roberts *et al.* (unpublished)
pC5106	Kan^r Spec^r Strep^r	42259	Unitary vector for plant transformation	Jefferson *et al.* (2006)
pC5105	Kan^r Spec^r Strep^r	49757	Unitary vector for plant transformation	Jefferson *et al.* (2006)

[a]Abbreviations: Gent^r, gentamicin; Tet^r, tetracycline; Chlor^r, chloramphenicol; Kan^r, kanamycin; Spec^r, spectinomycin; Strep^r, streptomycin.

http://www.ncbi.nlm.nih.gov/), with sequences obtained classified in 12 gene function families following their NCBI definition (Table 3).

Fourteen antibiotics were tested for their ability to inhibit the growth of *E. adhaerens* OV14, across three independent experiments with TTY media supplemented with kanamycin, neomycin, paramomycin, streptomycin, spectinomycin, carbenicillin, ampicillin, ticarcillin-clavunalate, cefotaxime or hygromycin-B at 0, 100, 200, 300, 400 or 500 mg L^{-1}. Chloramphenicol (0, 10, 25, 50, 100 or 200 mg L^{-1}), rifampicin (0, 25, 50, 75, 100 or 125 mg L^{-1}), gentamicin and tetracycline (0, 10, 20, 30, 40 or 50 mg L^{-1}) were also assessed with experiments conducted in a 48-well plate format for solid media assessment and 20 mL cultures (incubated at 28°C and 220 rpm) for liquid media evaluations. Visual observations of growth on agar and OD_{600nm} of liquid cultures were taken at 24-h intervals up to 120 h. An additional evaluation was also completed to determine the extent of kanamycin resistance at higher concentrations (0, 500, 1000, 1500, 2000, 2500 or 3000 mg L^{-1}) in *E. adhaerens* OV14.

Characterization of genetic elements supporting kanamycin resistance

A DELTA-BLASTp (Domain Enhanced Lookup Time Accelerated BLASTp) search was performed with the amino acid sequence of the aminoglycoside phosphotransferase (APH) domain (PF01636) accountable for kanamycin resistance in the Tn5 transposable element, against the *E. adhaerens* protein database (GenBank assembly accession: GCA_000583045.1) (Rudder *et al.* 2014) from NCBI using the default parameters (matrix BLOSUM62, existence 11 and extension 1). Protein sequences from *E. adhaerens* showing high levels of homology (*E*-value < 0.05) with the APH domain were recovered and aligned using the multiple sequence alignment software MultAlin (Corpet 1988).

For transcriptional investigations, the induction of expression by a kanamycin treatment (1000 mg L^{-1}) of the seven target genes identified in the sequence alignment (see Table S2, Supporting Information) was measured by RT-PCR (see Supplementary data for details on cell preparation, RNA extraction, cDNA synthesis, conditions of the RT-PCR assay and reference gene identification). Primer efficiencies were calculated using DNA dilution series for the reference and the target genes and Ct values obtained were dually normalized to expression of the *rpoA* reference gene.

Statistical analysis

All experiments were completed in triplicate with data collected into MS excel. Differences between treatments were identified (on experiments for media conditions and antibiotic phenotyping) using the REML correlation model for repeated measurements in GenStat v. 14.0. For the electroporation experiments, data were analysed using GenStat 14.0 with least significant differences ($\alpha = 0.05$) calculated to delineate among treatments applied post-ANOVA.

RESULTS

Mitigating aggregate formation in culture

Previous work with *E. adhaerens* OV14 cultured in LB broth (pH 7.0 ± 0.2, 220 rpm, 28°C) delivered erratic OD_{600nm} readings due to aggregate formation (illustrated in Fig. S1a, Supporting Information), which it was hypothesized diffused the spectrophotometric light source as it passed through the cuvette containing the culture volume. Of the eight media tested in this study, while the most rapid rate of growth was achieved in LB (Fig. S2, Supporting Information) up to 96 h ($P < 0.001$) this was achieved in the presence of aggregates. In contrast, both TY and TTY mitigated aggregate formation out to 120 h post-inoculation (illustrated in Fig. S1b, Supporting Information), with a faster growth rate of the two recorded with TTY (Fig. S2, Supporting Information). Separately, optimum pH (6.5), temperature (28°C) and rpm (220) parameters were identified as conducive for the production of homogeneous growth in TTY (data not shown, $OD_{600nm} = 0.95$, 96 h post-inoculation).

Propensity for electrotransformation

To facilitate the recovery of cells post-pulse, the expression medium used was changed from the standard SOC media to TTY, so that the volume and incubation time increased 10- and 4-fold, respectively. As a result incubating shocked cells into a larger volume of TTY and extending the incubation time significantly increased the transformation efficiency ($P < 0.001$). Transformed colonies were recorded for the smaller plasmids irrespective of voltage applied and/or incubation time but for the larger pCAMBIA plasmids, 2.5 Kv was significantly ($P < 0.001$) more effective than 1.8 Kv with an extended incubation time increasing efficiency 4-fold (Table 2). PCR analysis confirmed the presence of the respective plasmids in transformed strains with up to 15 colonies per plasmid evaluated (Fig. S3, Supporting Information).

Antibiotic profile of *E. adhaerens* strain OV14

In silico analysis identified 63 putative antibiotic resistance (AR) genes, classified into 12 different protein families (Table 3), with 15 of the 63 putative AR genes characterized as major facilitator super family (MFS)-type transporters. The remaining AR genes grouped into families characterized as haemolysins, H^+ antiporters, MarR and MarC domain proteins, glyoxolases, AcrB domain proteins, TfuA like domain proteins, ABC transporters, MATE-like domain proteins, EmrE and EmrA efflux and efflux pumps and RND transporters.

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Table 2. Transformation efficiency (CFU μg^{-1} DNA) of *E. adhaerens* OV14 electr-porated with plasmids of different sizes. Fisher's LSD are calculated at $\alpha = 0.05$ level.

			Transformants μg^{-1} of plasmid DNA			
		Recovery time (h)	1		4	
Bacterial genotypes	Plasmid	Voltage (Kv)	1.8	2.5	1.8	2.5
OV14	pRU1144		0	6.7×10^3	4.0×10^3	2.4×10^4
OV14	pRU1161		0	6.3×10^3	1.0×10^3	1.5×10^4
OV14_pC5105	pC2201		0	3.3×10^3	0	1.6×10^4
OV14	pC5106		0	1.0×10^3	0	4.3×10^3
$LSD_{Plasmid}$		d.f = 32, $n = 12$		1.3×10^3		
$LSD_{Voltage}$		d.f = 32, $n = 24$		0.9×10^3		
LSD_{Time}		d.f = 32, $n = 24$		0.9×10^3		
$LSD_{Plasmid \times Voltage}$		d.f = 32, $n = 6$		1.9×10^3		
$LSD_{Plasmid \times Time\ of\ recovery}$		d.f = 32, $n = 6$		1.9×10^3		
$LSD_{Plasmid \times Voltage \times Time\ of\ recovery}$		d.f = 32, $n = 3$		2.7×10^3		

This high diversity of putative AR genes identified was reflected in the broad antibiotic-resistant phenotype for *E. adhaerens* OV14 as confirmed for ampicillin, kanamycin, paramomycin, streptomycin, spectinomycin and ticarcillin–clavulanate (up to 500 mg L^{-1}, see Fig. S4a—j, Supporting Information). *Ensifer adhaerens* OV14 also demonstrated an ability to adapt to hygromycin-B and neomycin (<120 h incubation) and rifampicin (>120 h incubation) when cultured on solid media irrespective of the supplemented antibiotic concentration (see Fig. S4g, h, i, Supporting Information). While resistance to carbenicillin was recorded on solid media, this was not the case in carbenicillin supplemented liquid TTY media (see Fig. S4j, Supporting Information). *Ensifer adhaerens* OV14 was sensitive to gentamicin (≥10 mg L^{-1}), tetracycline (≥10 mg L^{-1}), chloramphenicol (≥50 mg L^{-1}) and cefotaxime (≥100 mg L^{-1}) in both liquid and solid media up to 120 h post-inoculation (see Fig. S4k, l, m, n, Supporting Information).

The extent of kanamycin resistance in *E. adhaerens* OV14 was confirmed with growth only impeded in TTY media supplemented with >2000 mg L^{-1} kanamycin. This contrasted with *E. adhaerens* OV14 + pC5105^{kanR}, which grew unimpeded on media supplemented with 3000 mg L^{-1} kanamycin after 120 h of inoculation (see Fig. S5, Supporting Information), which was equivalent to the included control strain of *A. tumefaciens* strain GV3101 harboring pC1305.2 ^{kanR}.

Identification and expression analysis of putative kanamycin resistance genes in *E. adhaerens* OV14

Within the *E. adhaerens* OV14 genome, seven proteins (AHK43653, AHK42618, AHK46053, AHK46036, AHK42883, AHK42277 and AHK42288) were identified with strong sequence similarity to the APH domain responsible for kanamycin resistance (Beck *et al.* 1982) (see Table S2, Supporting Information). Within the APH domain, seven sites exist grouping into ATP binding or antibiotic binding in function (Marchler-Bauer *et al.* 2015). Here, similarities (>40% identity) to all seven active sites (active site 1, 2, 3 and 5 for ATP binding; active site 4, 6 and 7 for antibiotic binding) were identified across the seven protein sequences (Fig. 1a).

Follow-up gene expression analysis of the seven putative kanamycin resistance genes identified *AHK42618* and *AHK42288* as the most responsive following 2 h exposure to 1000 mg L^{-1}, with a 1.7-fold and 4.6-fold increase, respectively (Fig. 1b). Beyond 4 h exposure, expression patterns were repressed across the identified targets with the exception of *AHK42288* which maintained a 'steady-state' level of expression as seen at time points –2 and 0 h (Fig. 1b).

DISCUSSION

The ability of *E. adhaerens* OV14 to accommodate horizontal gene transfer into plant genomes in the form of EMT has previously been highlighted (Wendt, Doohan and Mullins 2012). However, in contrast to the very broad microbiological and genetic understanding of *A. tumefaciens*, much less is known about *E. adhaerens* OV14, and *E. adhaerens* in general, lacks the level of original research required to develop this species as a useful tool for end users. In response, with this study we have focussed on addressing two basic requirements for *E. adhaerens* OV14: identifying optimal growth and electroporation conditions and characterizing its antibiotic profile.

Although the propensity of *E. adhaerens* OV14 to form aggregates (when grown in LB) did not appear to impede the overall functionality of EMT in delivering transgenic potato lines (Wendt, Doohan and Mullins 2012), this pleomorphic trait greatly complicated the logistics of trying to normalize OD_{600nm} recordings for the initial *E. adhaerens* OV14 starter culture. Such aggregate formation has been reported for *S. meliloti* (Szewczuk-Karpisz *et al.* 2014), a close relative of *E. adhaerens* and Young (2003) reported that *E. adhaerens* formed aggregates under adverse growth conditions due to its pleomorphic nature. In addition to identifying optimum pH, temperature and rpm conditions, the inclusion of a calcium-based salt in the growth media studied here minimized aggregate formation and afforded more consistent OD_{600nm} readings.

While the transformation efficiency of bacteria by electroporation is dependent on electric pulse and pulse rate (time constant) (Dower, Miller and Ragsdale 1988), preparing competent cells in TTY media delivered an uniform homogenous cell suspension in contrast to using a LB-based culture.

Of interest, a similar time of cell recovery was found to be beneficial for the electroporation of *Sinorhizobium* strains (Ferri *et al.* 2010). Although several reports detail the efficient transformation of *Rhizobium* species (Hayashi *et al.* 2000; Ferri *et al.* 2010), there are no specific detailed protocols for *E. adhaerens*. Taken

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Table 3. Classification into gene function families of putative 'antibiotic resistant genes' identified within the *E. adhaerens* OV14 genome.

Function	Accession number	Definition in NCBI
MFS transporter	AHK42355	Drug resistance transporter, EmrB/QacA subfamily
	AHK42487	Drug resistance transporter, EmrB/QacA subfamily
	AHK42756	Drug resistance transporter, Bcr/CflA subfamily
	AHK46148	Drug resistance transporter, Bcr/CflA subfamily
	AHK44795	MFS permease
	AHK44743	Putative tetracycline resistance protein
	AHK44391	Putative tetracycline resistance protein
	AHK44471	Drug resistance transporter, Bcr/CflA family
	AHK44472	Drug resistance transporter Bcr/CflA subfamily
	AHK45349	Drug resistance transporter, EmrB/QacA subfamily
	AHK46990	Drug resistance transporter, EmrB/QacA subfamily
	AHK47565	Drug resistance transporter, EmrB/QacA family
	AHK47615	Drug resistance transporter, EmrB/QacA subfamily
	AHK43880	Drug resistance transporter, EmrB/QacA subfamily
	AHK43005	Drug resistance transporter, Bcr/CflA subfamily
Haemolysin D	AHK42488	Putative multidrug resistance protein
	AHK45377	Putative multidrug efflux transporter protein
H^+ antiporter	AHK42504	Multiple resistance and pH regulation protein F
	AHK42502	Multiple resistance/pH regulation related protein D
MarR and MarC domain>	AHK43715	Putative MarR family transcriptional regulator
	AHK44477	Putative MarR family transcriptional regulator
	AHK42757	Putative transcriptional regulator, MarR family
	AHK42415	Putative transcriptional regulator, MarR family
	AHK42729	Putative transcriptional regulator, MarR family
	AHK43090	Transcriptional regulator, MarR family
	AHK43095	Putative transcriptional regulator, MarR family
	AHK47248	Putative MarC family transmembrane protein
	AHK44413	Putative MarC family transmembrane protein
	AHK42416	Putative transcriptional regulator, MarR family
Glyoxolase	AHK42781	Glyoxalase/bleomycin resistance protein/dioxygenase superfamily
	AHK42959	Putative glyoxalase/bleomycin resistance protein/dioxygenase
	AHK45250	Putative glyoxalase/bleomycin resistance protein/dioxygenase
	AHK43500	Hypothetical protein
	AHK43179	Putative glyoxalase/bleomycin resistance protein/dioxygenase
	AHK45252	Glyoxalase/bleomycin resistance protein/dioxygenase
	AHK46351	Putative glyoxalase/bleomycin resistance protein/dioxygenase
	AHK42578	Putative glyoxalase/bleomycin resistance protein/dioxygenase
	AHK43079	Putative glyoxalase/bleomycin resistance protein/dioxygenase
	AHK44857	Putative glyoxalase/bleomycin resistance protein/dioxygenase
	AHK44622	Glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein
AcrB domain	AHK45378	Component of multidrug efflux system
	AHK47157	Component of multidrug efflux system
TfuA-like domain	AHK45520	Hypothetical protein
	AHK46994	Hypothetical protein
ABC transporter	AHK44854	Putative branched-chain amino acid permease (azaleucine resistance) protein
MATE-like domain	AHK47570	DNA-damage-inducible protein F
	AHK44390	Putative multidrug efflux protein
EmrE and EmrA efflux and efflux pumps	AHK45178	Quaternary ammonium compound-resistance protein QacE
	AHK46991	Putative multidrug resistance protein
	AHK47499	Multidrug resistance protein A
	AHK47566	Multidrug resistance protein A
	AHK44363	Putative multidrug transport protein
	AHK44364	Putative multidrug efflux system transporter
	AHK46009	Multidrug resistance protein A
	AHK47355	Multidrug resistance protein A
	AHK46009	Multidrug resistance protein A
RND transporter	AHK47155	Putative multidrug efflux transporter protein
	AHK47156	Putative multidrug efflux transporter protein
	AHK42546	Cation/multidrug efflux pump, RND superfamily
	AHK45220	Cation/multidrug efflux pump, RND superfamily
	AHK42341	RND multidrug efflux transporter MexF
	AHK47037	RND multidrug efflux transporter MexF
Unknown function	AHK43808	Putative transmembrane protein

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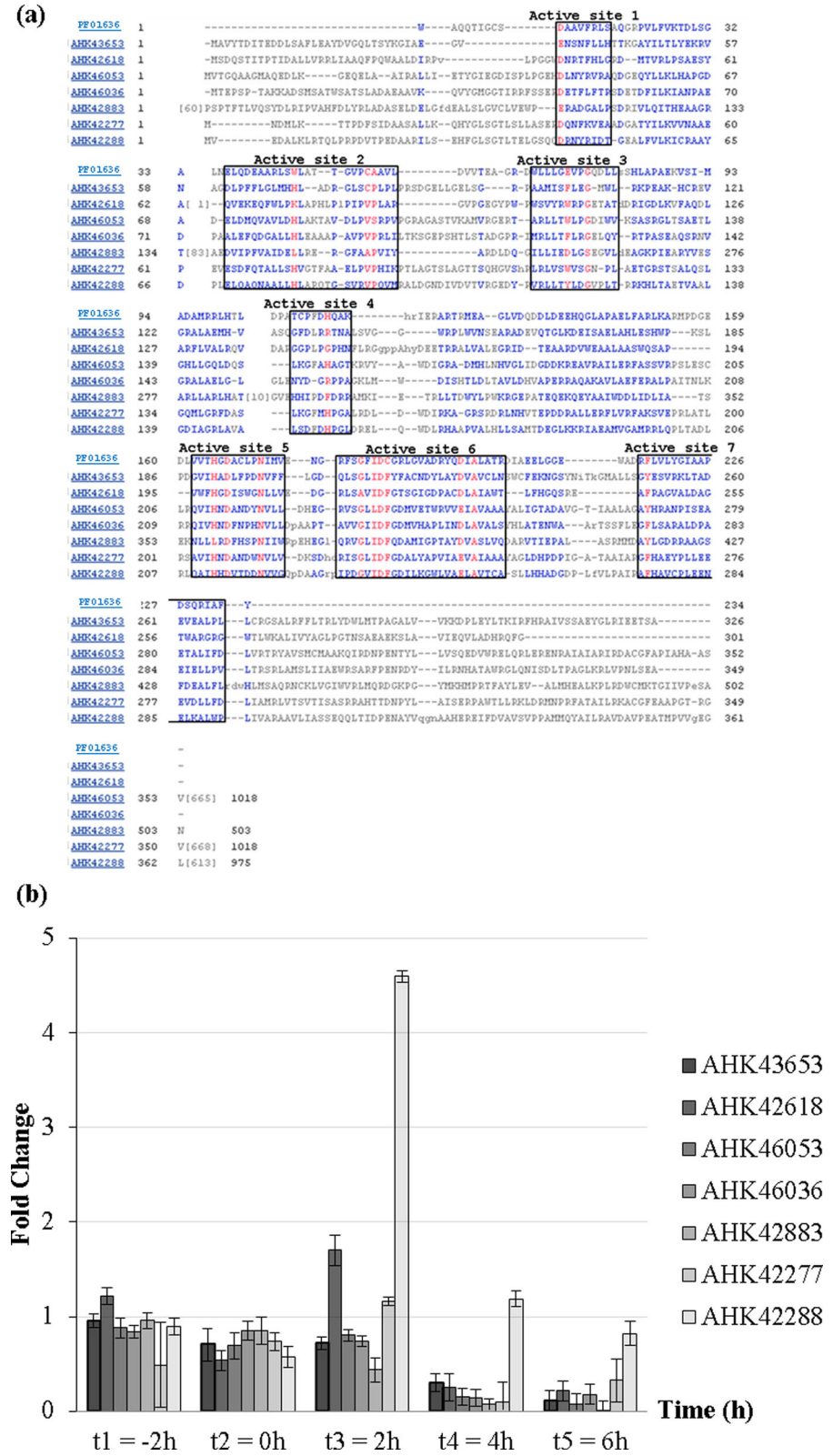

Figure 1. Protein sequence alignment and expression analysis of putative kanamycin resistance genes in *E. adhaerens* OV14. (**a**) DELTA-BLAST on *E. adhaerens* genome using the amino-acid sequence of the APH domain (PF01636). Seven proteins in *E. adhaerens* OV14: AHK43653, AHK42618, AHK46053, AHK46036, AHK42883, AHK42277 and AHK42288 showed high levels of homology with the APH domain. Multiple sequence alignment columns with no gaps are colored in blue or red. Red color indicates >60%, blue color >40% conserved domain sequence, black columns = 0% conservation. Domains 1, 2, 3 and 5: ATP-binding site. Domains 4, 6 and 7: antibiotic-binding site. (**b**) Expression of putative kanamycin-resistant genes: *AHK43653*, *AHK42618*, *AHK46053*, *AHK46036*, *AHK42883*, *AHK42277* and *AHK42288* in *E. adhaerens* OV14 following a kanamycin stress (1000 mg L^{-1}) at t2 = 0 h.

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together, the effect of these modifications to the protocol significantly increased the transformation efficiency of *E. adhaerens* OV14 to the point where combined plasmid content (pC5105 + pC2201) of up to 53Kb was attained and as such the electrotransformation of alternative strains of *E. adhaerens* should be possible employing the conditions detailed here.

The *in silico* search for AR genes across the genome of *E. adhaerens* OV14 further supports the conclusion that soil bacteria are a large source of antibiotic-resistant genes (Riesenfeld, Goodman and Handelsman 2004). With 63 potential AR genes in *E. adhaerens* OV14 it could be hypothesized that the strain has accumulated this number through its tendency for prokaryotic predation, as previously reported (Casida (1982); yet recent studies indicate the high diversity of AR genes within soil bacteria communities (Forsberg *et al.* 2012, 2014). Regarding *Ensifer* spp., previous studies performed by Wang *et al.* (2002), Young (2003) and Bromfield *et al.* (2010) reported carbenicillin, kanamycin and neomycin resistance in *Ensifer* sp., with Rogel *et al.* (2001) and Willems *et al.* (2003) reporting the sensitivity of certain *Ensifer* sp. to gentamicin and tetracycline, respectively. While informative the conclusions from these studies are partial. In contrast, the *in silico* and *in vitro* antibiotic profile of *E. adhaerens* OV14 completed in this study provides a solid knowledge base for present and future users of EMT and may also be of interest to those research groups working on alternative strains of *E. adhaerens*. More specifically, the response of *E. adhaerens* OV14 is significant owing to the dependence of kanamycin resistance as a prokaryotic marker on a large number of plant transformation vectors. However, by identifying the most transcriptionally active nptII type homologs in *E. adhaerens* OV14 in this study, it should now be possible to edit the strain's genome via Cas9 editing (Cong *et al.* 2013) in order to generate a kanamycin-sensitive phenotype and hence broaden the applicability of *E. adhaerens* OV14 for current and future users.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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Conflict of interest

E. Mullins and F. Doohan are authors of patent application PCT/EP2010/070681 which details the use of an isolated *Ensifer adhaerens* strain OV14 deposited under NCIMB Accession Number 41777 as a gene delivery system in the genetic transformation of plant material. Our manuscript has in no way been affected by this fact, nor has our participation in the work influenced in any manner the analysis of the generated datasets and/or the conclusions drawn.

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