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Identification of Genes Required for Soil Survival in *Burkholderia thailandensis* by Transposon-Directed Insertion Site Sequencing

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Abstract Transposon-directed insertion site sequencing was used to identify genes required by *Burkholderia thailandensis* to survive in plant/soil microcosms. A total of 1,153 genetic loci fulfilled the criteria as being likely to encode survival characteristics. Of these, 203 (17.6 %) were associated with uptake and transport systems; 463 loci (40.1 %) coded for enzymatic properties, 99 of these (21.4 %) had reduction/oxidation functions; 117 (10.1 %) were gene regulation or sensory loci; 61 (5.3 %) encoded structural proteins found in the cell envelope or with enzymatic activities related to it, distinct from these, 46 (4.0 %) were involved in chemotaxis and flagellum, or pilus synthesis; 39 (3.4 %) were transposase enzymes or were bacteriophage-derived; and 30 (2.6 %) were involved in the production of antibiotics or siderophores. Two hundred and twenty genes (19.1 %) encoded hypothetical proteins or those of unknown function. Given the importance of motility and pilus formation in microcosm persistence the nature of the colonization of the rhizosphere was examined by confocal microscopy. Wild type *B. thailandensis* expressing red fluorescent protein was inoculated into microcosms. Even though the roots had been washed, the bacteria were still present but they were motile with no attachment having taken place, perhaps being retained in a biofilm.

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Introduction

The genus *Burkholderia* comprises bacteria with a wide range of ecological niches [5]. Some species are plant pathogens, while others promote plant growth by nitrogen fixation; members of the *Burkholderia cepacia* complex may be exploited for bioremediation while also being opportunistic human pathogens. The only primary pathogens of humans in the genus are *Burkholderia mallei* and *Burkholderia pseudomallei.* Most of the knowledge about these organisms gained to date concerns their pathogenicity. It is known that *B. mallei* does not survive in the environment and has never been isolated other than from an animal host, predominantly equines [20, 25]. *B. pseudomallei* can survive in the environment [25, 32] where its natural range is Southeast Asia, Northern Australia, South and Central America and the Middle East [25]. It can colonize not only the rhizosphere but also the leaves of some grasses and is, thus, increasing its geographical range and potential for human infection [23]. The genetic defects in *B. mallei* that preclude its growth outside of a host result from a reduced genome size compared to its close relative. This has been attributed to an insertion sequence-mediated process [33]. The human pathogenicity of *B. mallei* and *B. pseudomallei* has made them regarded as the agents of concern as potential biological warfare agents (BWAs) [42].

A close relative of the two potential BWAs is *Burkholderia thailandensis* which is non-pathogenic and a soil inhabitant [13, 25]. It has been isolated in Southeast Asia, Australia, and America [13]. As a result of the close genetic relatedness of *B. thailandensis* [5] it is a useful surrogate for the potential BWAs. From a comparison of the complete genomes of the three species, it is evident that there is a high degree of conservation [25]. The similarity

between the genomes of *B. pseudomallei* and *B. thailandensis* extends to a surprising degree. Although Type Three Secretory Systems [6] are required for pathogenesis by many Gram-negative bacteria this cluster of genes is extensively conserved in the non-pathogenic *B. thailandensis* [25], suggesting a role in environmental survival.

A striking feature of the architecture of the *B. thailandensis* genome is the presence of at least 15 regions of either atypical GC content or regions of 'phage-related genes and 'phage-like integrases [51]. These genomic islands are not found in *B. pseudomallei* or *B. mallei*, presumably having emerged after the divergence of these two pathogens. In spite of this there is greater conservation of gene order between *B. thailandensis* and *B. pseudomallei* than there is between the latter and *B. mallei*. This syntenic conservation is also considerably higher than in many other pairs of virulent and non-virulent pairs of bacterial species [51]. The functional constraint acting as a negative selective force to prevent extensive genomic reorganization may be the necessity of both to survive in the environment. Part of the reason for the inability of *B. mallei* to exist on a non-host environment seems to be its sensitivity to extreme environments, including heat and desiccation [45, 48]. It lacks functional flagella, due to mutations in a few key genes, even though it has retained most of the genes for motility and chemotaxis. *B. pseudomallei*, on the other hand, is an opportunistic pathogen.

There is a great value in understanding the mechanisms that allow bacteria to persist in the environment, be they potential BWAs, promoters of plant productivity or plant pathogens. Very few reports exist of whole genome screens for genes essential to environmental survival. Those that exist have used signature tagged mutagenesis (STM) followed by hybridization to identify mutants not surviving an environmental challenge [8, 14, 29, 36]. A new approach has been devised which exploits the power of high-throughput sequencing and which can be described as Transposon Directed Insertion-Site Sequencing (TraDIS) [12, 27, 47]. Like STM, it is a negative selection method. An undefined library of bacteria is made with the saturation coverage of transposon insertions into the genome. This is termed the input pool which is then subjected to a survival challenge. The population of mutants subsequently recovered is called the output pool. Deep sequencing of the region adjacent to the transposon insertion site is carried out for both the input and output pools. By identifying the genetic loci present in the former, but not the latter one can identify those genes required for survival: a gene is absent or present in decreased frequency in the output pool because the bacteria carrying it had a transposon-disrupted and, hence, non-functional copy. It is thus possible to combine the screening for non-surviving mutants with the concomitant identification of the genes where those mutations lie. Here, we used TraDIS to identify the genes required for *B. thailandensis* to survive in a non-axenic plant/soil microcosm. This can inform on the persistence of *B. pseudomallei* and give insights into why *B. mallei* is an obligate pathogen.

Materials and Methods

Bacterial Strains, Media, and DNA Vectors

The strain of *B. thailandensis* used (DSM13276) is an environmental isolate [3] and was purchased from DSMZ (Braunschweig, Germany). A selection of STM mutants (code numbers: 2-C5, 1-G5, I-G4, 3B-10) of *B. vietnamiensis* unable to colonize a pea rhizosphere model [36] and the wild type strain, G4, were a kind gift from Dr. Mahenthiralingam (University of Cardiff, UK). Routine culturing used Tryptone Soy Agar (TSA) and Tryptone Soy Broth (TSB) (Oxoid). The minimal media used was M9 with glucose [38].

The transposon-containing plasmid (pHBurk3) used to generate the saturation library was a gift from Prof. Herbert Schweizer (Colorado State University, USA) [38]. A plasmid (pBHR-pGros-RFP) capable of expressing red fluorescent protein (RFP) in *B. thailandensis* was a gift from Dr Andrew Scott (Dstl, UK).

Electroporation and Transposition

The bacteria were incubated after electroporation [44] in Brain Heart Infusion (BHI) broth (Oxoid) containing 0.5 % glycerol (Sigma, UK) for 90 min at 30 °C, with shaking, before plating onto BHI agar containing kanamycin (50 μg/ml) for pBHR-pGros-RFP and zeocin (200 μg/ml) for pHBurk3. Cells transformed with pHBurk3 were cultured overnight at 37 °C, the nonpermissive temperature for plasmid replication [38]. Cells which had lost the plasmid, but maintained the integrated transposon were susceptible to zeocin but maintained kanamycin resistance (500 μg/ml). Verification that random transposition had occurred in eight clones with this phenotype was obtained as previously described [38]. It was assumed that a saturation library of transposition events into the genome had occurred in this population of bacteria as a whole.

Microcosms

Bacteria were inoculated into soil (8 g approximately) contained in 24 wells (3.5 cm diameter) in multiwell plates

(Nunc). The soil used for *B. thailandensis* was a sandy loam taken from the top 10 cm of soil in Northern Thailand (Dr. Nuttakan Nitayapat, Kasetsart University, Bangkok, Thailand). Its composition was clay (3 %), silt (10 %), sand (87 %) and organic matter (2 %) with a pH value of 4.8 (Forestry Commission Soil Science Laboratory, UK). The following plants (Emorsgate Seeds) used were: *Avenula pratense* (meadow oat grass), *Dactylis* glomerata (cocksfoot), *Festuca pratensis* (meadow fescue), *Festuca rubra* (red fescue) (*Lolium perenne* (ryegrass), *Schedonorus arundinacea* (tall fescue), *Phleum pratense* (Timothy) and *Trifolium pratensis* (red clover). Seeds were germinated and grown in non-sterile tap water to a root length of about 2 cm. Ten seedlings, two of each species, were transplanted per well and soil (approximately 8 g) added. The soil was then inoculated with a bacterial suspension. Distilled water was added to moisten the soil and the plates were then incubated at 20 °C, 12:12, light:dark photoperiod and 80 % humidity in an environmental cabinet (Weiss Gallenkamp).

Preparation of Bacteria for Microcosm Inoculation

The saturation library of bacteria, above, was passaged several times through M9 medium with glucose. The aim was to remove mutants in central metabolic pathways, not directly related to survival in the soil, but which might have survived growth in a rich medium. This population of bacteria was termed "the input pool." Prior to inoculation into the microcosms, these bacteria were grown statically at room temperature in 75:25 % M9:TSB, overnight. The early vegetative phase bacteria were quantified spectrophotometrically, harvested by centrifugation at 6,000×*g* and washed in PBS.

Recovery of Bacteria from the Microcosms

After 2 weeks the microcosms were harvested by shaking the contents of entire wells in soil extraction buffer (0.5 % Tween 20 and 0.1 % sodium pyrophosphate) to a total volume of 45 ml for 20 min at setting 8 on a random motion oscillator (Gallenkamp). To enumerate bacteria, serial dilutions were made in sterile PBS and plated in duplicate onto TSA containing kanamycin (500 µg/ml). The slurry (8 ml) obtained from shaking the soil with extraction buffer was loaded onto 5 ml of Histodenz (Sigma), made to a density of 1.3 g/ml, in 15 ml tubes (Falcon). These were centrifuged at 5,000×*g* for 1.5 h. The layer containing the bacteria was aspirated with a sterile Pasteur pipette.

TraDIS Procedures

Genomic DNA was initially extracted from the input and output pools of *B. thailandensis* using the Pure Gene kit (Qiagen). Each DNA pool was fragmented by nebulisation (30 psi nitrogen for 6 min using an Invitrogen nebuliser) and the resulting fragments size-assessed on an Agilent Bioanalyser High Sensitivity chip. Fragmented DNA was resolved on a 2 % (w/v) agarose gel, and DNA in the size range 250–400 bp excised and purified using a QiaExII gel extraction kit (Qiagen). The fragment libraries were end-polished and A-tailed using a NEBNext DNA library preparation kit for the Illumina sequencing (New England Biolabs), ready for adapter ligation. Double stranded adapters Ind_Ad_T and Ind_Ad_B (Table 1) were annealed and ligated to the fragment libraries. These were quantified by qPCR using the primers Ad_T_qPCR1 and Ad_B_qPCR2 (Table 1) using an Illumina library quantification kit (KAPA Biosystems), according to the manufacturer's instructions.

Adapter-ligated fragments were then enriched for those specifically containing a transposon insertion site using PCR primers with homology to the 3′ inverted repeat sequence of pAW068. The PCR thermal cycle was as follows: 90 °C for 30 s, followed by 24 cycles of 98 °C for 10 s; 65 °C for 30 s; and 72 °C for 30 s. The first four cycles contained only the transposon-specific primer to maximize enrichment of the transposon-associated fragments. The transposon specific primer, pHBurk3-p5 (Table 1), also contained the Illumina P5 end for attachment to the Illumina flow cell. The reverse primer, RInV3.3 (Table 1) which contained the Illumina P7 end, was added at the start of the fifth cycle. To limit PCR bias, multiple PCR reactions were run in parallel and pooled after size selection to generate sufficient concentrations of library material. PCR products were resolved on a 2 % (w/v) agarose gel. Successfully amplified products were purified and size-fractionated by gel electrophoresis and the DNA recovered using a QiaExII gel extraction kit (Qiagen). DNA was eluted in 20 µl elution buffer and quantified by qPCR (KAPA Biosystems). Sequencing was performed by the University of Exeter Sequencing Service on an Illumina HiSeq 2500 instrument and analyzed using Integrative Genomics Viewer (IGV) software [40].

Visualization of Bacteria in the Rhizosphere

Microcosms were inoculated as above but using *B. thailandensis* containing pBHR-pGros-RFP. After one week, the seedlings were carefully pulled from the soil and washed in PBS. The roots were excised and mounted on a slide for examination by confocal microscopy (Zeiss Observer Z1).

Table 1 PCR primers and oligonucleotides, the asterisk denotes a phosphorothioate base

Primer name	DNA sequence (5′–3′)
Double stranded adaptor Ind_Ad_T	ACACTCTTTCCCTACACGACGCTCTTCCGATC*T
Double stranded adaptor Ind_Ad_B	GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTC
Quantitative PCR primer Ad_T_qPCR1	CTTTCCCTACACGACGCTCTTC
Quantitative PCR primer Ad_B_qPCR2	ATTCCTGCTGAACCGCTCTTC
Transposon -specific PCR primer for pHBurk3-p5 library preparation primer	AATGATACGGCGACCACCGGGGGACTTATCAG CCAACCTG
Reverse sequencing primer RInV3.3 for pHBurk3	CAAGCAGAAGACGGCATACGAGATCGGTACACTCTTTCCCT ACACGACGCTCTTCCGATCT
pHBurk3-p5 sequencing primer	AATGATACGGCGACCACCGGGGGACTTATCAG CCAACCTG

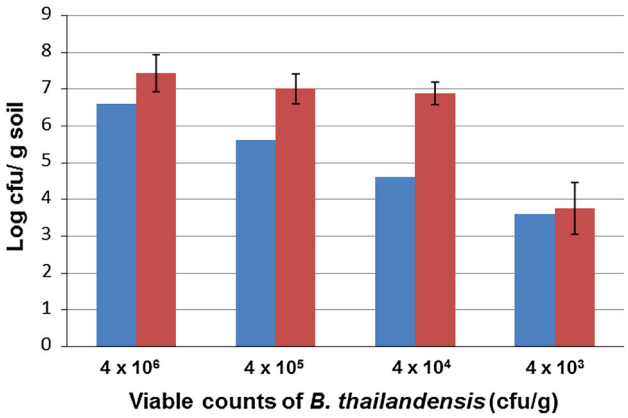

Fig. 1 Level of colonization of plant/soil microcosms by *B. thailandensis*. *Dark grey* Initial inoculum (cfu g^{-1} soil). *Light grey* Final cell density (cfu g^{-1} soil) after incubation for 3 weeks. The experiment was performed three times with six replicate wells for each dilution on each occasion. *Error bars* represent standard deviation

Results and Discussion

Inoculation of Microcosms

Perhaps the most crucial aspect of carrying out negative selection procedures such as TraDIS is insuring that the bacteria are subjected to a stringent survival challenge. Microcosms were inoculated with the increasing numbers of *B. thailandensis* cells and the final density assessed after incubation for 2 weeks. It is evident from Fig. 1 that similar final densities of *B. thailandensis* were achieved from initial inoculum densities of 4×10^4–4×10^6 CFU/g soil. When a further ten-fold lower inoculum density was used, a noticeably lower and more unpredictable recovery resulted (Fig. 1). The lowest inoculum level capable of producing a reliable colonization of the microcosms (4×10^4 CFU/g soil) was, therefore, chosen. It is important to avoid so-called stochastic errors or selection bottlenecks [10, 30]: mutants that might have been capable of persisting through the challenge are lost simply because too many bacteria were initially inoculated. Equally, attenuated bacteria survive because they did not experience a sufficiently stringent survival challenge. This results in random loss or survival in the output population. Sufficient bacteria were harvested in the output pool to provide greater than a hundred-fold increase beyond the input pool to expect, at the 95 % confidence interval, that a particular mutant has been lost due to failure to survive as opposed to chance [9]. Grasses were chosen as the plant species to populate the microcosms because they are easy to grow, their roots are readily colonized by *B. thailandensis* and grasses are important to the ecology of *B. pseudomallei* [23].

An STM library of *Burkholderia vietnamiensis* was previously used to study rhizosphere colonization in a pea model [36]. As a means of verifying the stringency of the conditions developed here four of those mutants of *B. vietnamiensis* that were unable to colonize pea roots were inoculated into the microcosm. None of the mutants was recoverable at the minimum detection level (10^3 CFU/g soil) after 2 weeks. The wild type strain, however, produced a colonization level similar to that of *B. thailandensis* (data not shown). Although the two *Burkholderia* species are not closely related [5] the finding strengthened the confidence in the stringency of the challenge that *B. thailandensis* was subjected to.

Analysis of Genes Required for Survival in the Microcosms

The genome of *B. thailandensis* E264 is 6.72 Mb in size and contains a predicted 5,713 protein-coding genes (http://www.ebi.ac.uk/ena/data/view/Project:PRJNA10774). By comparing it with the TraDIS output from the environmental strain of *B. thailandensis* used here, 1,153 genetic loci were identified as being important for survival in the plant/soil environment. Among the interesting groups of loci appearing were: 203 (17.6 %) associated with uptake and transport systems (Supplementary Information, Table 1); 463 loci (40.1 %) coded for enzymatic

properties, of which 99 (21.4 %) had oxidation/reduction functions (Supplementary Information, Table 2); 117 (10.1 %) were sensory and genetic regulatory loci (Supplementary Information, Table 3); 61 (5.3 %) encoded structural proteins found in the cell envelope or with enzymatic activities related to it (Supplementary Information, Table 4), distinct from these, 46 (4 %) were involved in chemotaxis and flagellum or pilus synthesis (Supplementary Information, Table 5); 39 (3.4 %) were transposase enzymes or are bacteriophage-derived (Supplementary Information, Table 6); 30 (2.6 %) concerned products with antibiotic or siderophore activity or conferred resistance to toxic compounds (Supplementary Information, Table 7). Two hundred and twenty genes (19.1 %) encoded hypothetical proteins or those of unknown function. The overwhelming impression from these major categories of attenuated genes, above, is the struggle for survival that existed in the microcosms: the necessity, for example, to scavenge for nutrients, control gene expression and synthesize antibiotics, while protecting against those produced by others, illustrates an ongoing struggle for persistence. This is strong evidence that the the microcosm conditions developed were a stringent survival challenge.

The 'survival factor' given in the tables of genes required for persistence (Supplementary Information) was obtained by dividing the number of insertion sites in a gene found in the input pool by the respective number in the output pool. Statistical analysis [10] has shown that a minimum of 32 sequence reads is required in the input pool for a given gene locus to be confident that any decrease in frequency in the output pool is significant. Furthermore, the same study demonstrated that a two-fold decrease in the frequency of a gene locus in the ouput pool compared to the input is the threshold level that a transposon insertion has resulted in a loss of fitness. These criteria were adopted for the data presented here.

Transport Systems

Transport mechanisms were the most numerous group of survival genes identified (Supplementary Information, Table 1). Their functions ranged from the uptake of ions to the export of proteins. The difficulty in making ecological interpretations of the data found is illustrated by putrescine (1,4-diaminobutane). There were five permease proteins that transport this polyamine identified in Table 1 (Supplementary Information). Furthermore, an enzyme directly required for its synthesis (agmatinase, BTH_II0784) was also a persistence characteristic (Supplementary Information, Table 8). It has long been known that putrescine was involved in osmotic control in Gram-negative bacteria, but it is becoming evident that it plays highly varied roles such as: incorporation into the cell envelope; acid resistance, free radical scavenging; siderophore synthesis; and biofilm formation [50]. Discerning which of these functions putrescine is required for at any one time is a difficult undertaking.

There are several Type Two and Three Secretion System proteins encoded by the survival genes in Table 1 (Supplementary Information). Although these are required for pathogenesis by many Gram-negative bacteria [6] this cluster of genes is extensively conserved between the non-pathogenic *B. thailandensis* and the pathogens *B. mallei and B. pseudomallei* [25]. The function of these proteins that makes them necessary for soil survival is unknown, although an ortholog of a Type Three Secretion System chaperone to be a fitness factor for environmental survival in the anaerobe *Shewanella oneidensis* [8]. More understandable are the Type VI Secretion System proteins (Supplementary Information, Table 1). Their functions include bacterial cell targeting, conjugation, gene regulation, and cellular adhesion [41].

Sensory and Regulatory Genes

Over 10 % of the 1,153 genes identified as being required for soil survival were genes for regulatory or sensory mechansims. Most of these are listed in listed in Table 3 (Supplementary Information) with, where known, the function to which they are connected. In addition to these are a number of regulatory genes involved in motility that are listed in Table 5 (Supplementary Information). Teleologically it is easy to understand the need to control metabolic and biosynthetic pathways in a nutrient-limited, variable, and highly competitive environment. Genome analysis of one strain of *Arthrobacter* spp., a frequently isolated soil bacterium, discovered a disproportionately a high number of one- and two-component regulatory genes [35]. These were hypothesized to contribute to its ubiquity in soil.

Energy Generation and Carbon Metabolism

Key indicators that nutrient supply was not plentiful is the attenuation in survival caused by mechanisms orchestrating the response to limited carbon levels such as starvation-inducible DNA-binding protein, DpsA (BTH_I1284) and stringent starvation protein A (BTH_I2974). A further indicator that *B. thailandensis* was scavenging small organic molecules as carbon sources was the appearance as survival characteristics of genes *aceA* (BTH_II2194), *aceB* (BTH_II2193), and *aceK* (BTH_II2197) which encode key enzymes in the glyoxylate pathway (Supplementary Information, Table 8). Similarly, the gene for alanine-glyoxylate aminotransferase (BTH_I1404) was a survival factor (Supplementary Information, Table 8). This enzyme enables

bacteria to use glycine as a carbon source, particularly in oligotrophic conditions [49]. In the quite closely related *Ralstonia eutropha*, glyoxylate reductase (the gene for which, BTH_I0123, was also significantly diminished in the *B. thailandensis* output pool) is important for acetate metabolism [4]. It is noteworthy that this can originate from the environment or from internal poly 3-hydroxybutyrate degradation. For *B. thailandensis*, several enzymes involved in poly 3-hydroxybutyrate metabolism were soil persistence genes (3-hydroxybutyrate dehydrogenase, BTH_II2038; poly 3-hydroxybutyrate depolymerase, BTH_I2713; 3-hydroxybutyrate oligomer hydrolase, BTH_I1420; acetoacetyl CoA reductase, BTH_I2257; and polyhydroxylalkanoic acid synthetase, BTH_I2255) (Supplementary Information, Table 8). When *B. thailandensis* was cultured solely in minimal medium, immediately prior to inoculation into the microcosms, the resulting colonization was, at best, poor. Bacteria inoculated from stationary phase rather than exponential phase cultures also resulted in poorer colonization (data not shown). This implies that stored carbon and energy stores were insufficient on their own and that flow into and out of energy reserves during growth in the soil was part of a 'feast and famine [7] life-style common to nearly all bacteria. It has previously been shown [28] that a mutant deficient in 3-hydroxybutyrate storage survived, as well as the wild-type strain in sterile soil but produced a 3.5-fold lower cell density in non-sterile soil. Storage compounds are obviously a vital mechanism in competition with other soil micro-organisms.

Bacteriophage-Related Genes

Particularly striking in *B. thailandensis* is that 3.4 % of the loci identified are transposases or 'phage-derived. Genetic fluidity has previously been recognized as an important factor in *B. pseudomallei* [18]. Others have also presented evidence that the genome of *B. pseudomallei* is highly plastic and was specifically biased toward genes involved in mobile elements, among others [42]. Many bacteria contain genomic islands and transmissable elements that endow them with pathogenicity and environmental survival characteristics [18]. These TraDIS data imply that genetic reorganization might be an important factor in environmental survival. Another role for 'phage elements is in the formation of biofilms [22]. The role of these loci in environmental survival merits further study.

Motility

Twenty four survival genes were flagellar proteins or related to chemotaxis (Table 5, Supplementary Information). Although, evidently, not required for growth in vitro, the role of motility in the rhizosphere was examined using

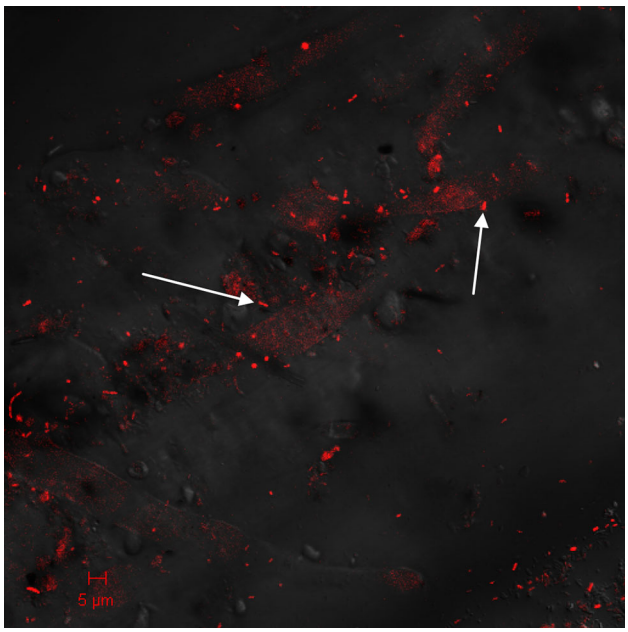

Fig. 2 Confocal micrograph of *B. thailandensis* (*white arrows*) containing pBHR-gros-RFP plasmid. The bacteria were allowed to colonize soil microcosms containing grass seedlings for 1 week prior to extraction and washing of the roots. The *llabeled bacteria* were freely motile in the rhizosphere and none were observed to be attached to the roots or root hairs

RFP-labelled *B. thailandensis*. Grass roots extacted from the soil after 1 week of incubation and washed in phosphate-buffered saline (PBS) were examined by confocal microscopy (Fig. 2). Fluorescent bacteria were clearly visible in the rhizosphere but, crucially, none were observed to be attached to the roots and all were motile. In view of the fact that the roots had been washed, it was assumed that the bacteria were kept in the vicinity of the root in a biofilm. The secretion by roots of sugars, amino acids, proteins etc. [1] are the most important reason for their colonization by micro-organisms. The benefit of remaining in this region while being able to respond to positive or negative stimuli may be part of the survival strategy of *B. thailandensis*. It has been suggested that biofilms are an important feature of the environmental survival of *B. pseudomallei* [26]. Motility was identified as a colonizing factor for *Pseudomonas* spp. in a gnotobiotic rhizosphere model [43], while the phenomenon of bacteria swimming through holes in biofilms has recently been reported [19].

Antibiotic and Siderophore Production

A fascinating insight into the existence of bacteria in soil is given by the dependence of *B. thailandensis* on the production of and resistance to toxic compounds. As might be expected, antibiotic production has been shown to have an

Table 2 Cluster of genes identified by TraDIS in *B. thailandensis* involved in siderophore production

Gene accession number	Survival factor	Encoded proteins
BTH_I2367	9.00	Dihydroaeruginoic acid synthetase
BTH_I2366	6.76	Polyketide synthase
BTH_I2365	2.81	Polyketide synthase
BTH_I2364	2.19	Peptide synthetase
BTH_I2363	2.11	Polyketide synthetase
BTH_I2362	N.S.	AcylCoA dehydrogenase
BTH_I2361	N.S.	Phosphotransferase
BTH_I2360	10.00	Non-ribosomal peptide synthetase, putative
BTH_I2359	N.S.	Pyridine nucleotide disulfide oxidoreductase

Genes not identified by TraDIS are signified as 'Not Significant' (N.S.)

important effect on rhizosphere community structure [24]. Proteins conferring resistance to several types of antibiotic are shown in Table 7 (Supplementary Information). In addition to these, several of the transport proteins identified in Table 1 (Supplementary Information) relate to antibiotic efflux pumps. Of course, efflux pumps that function as resistance mechanisms may also have a role in detoxification of intracellular metabolites, cell homeostasis, and intercellular trafficking [34]. Some of the genes in Table 7 (Supplementary Information) refer to the synthesis of polyketides and non-ribosomal peptides. Both of these types of product can be involved in antibiotic and siderophore production [21, 39]. The latter, which are involved in metal ion uptake, can be considered to be an adjunct to the many nutrient uptake systems listed in Table 1 (Supplementary Information). One cluster of genes that is predicted in silico to form an operon is detailed in Table 2 and illustrated in Fig. 3. It contains the dihydroaeruginoic acid synthetase gene that is central to the siderophore pyochelin produced by the quite closely related *Pseudomonas aeruginosa* [37]. It is important to note that not all the genes in the predicted operon were identified by TraDIS. The missing genes are indicated as "Not significant" in Table 1 and by black arrows in the Fig. 3. This phenomenon was reported previously: only about half of the genes present in the locus of enterocyte effacement, which is a crucial pathogenesis operon in *Escherichia coli*, were identified by TraDIS [10]. The reasons behind this may include incomplete genome coverage and too few insertions in a genetic locus to satisfy the statistical thresholds for significance. Nevertheless, the IGV software demonstrated that a saturation library in the input pool had been produced. It is also of interest that, while not greatly dissimilar, there is a 5-fold difference in the survival factors of the genes in Table 1. With the recognition that micro-organisms rarely exist in nature outside of communities and biofilms [15], it is possible that complementation may occur for a bacterium attenuated in the production of secreted products such as an antibiotic.

Of the limited number of comparable, environmental studies STM has been used to identify genes required by *Desulfovibrio desulfuricans* [29] and *S. oneidensis* [14] to survive in aquifer sediments. These are sulfur-reducing and metal-reducing bacteria, respectively, which grow under anaerobic conditions. Of 97 open reading frames identified in *D. desulfuricans* the major groups of functions favoring survival included: transport and nutrient binding; signal transduction; transcriptional regulation; energy generation; amino acid biosynthesis, carbohydrate and lipid metabolism; and cell envelope synthesis. Notably, also, several transposases and bacteriophage-related proteins were identified. For *S. oneidensis*, the 47 survival genes included those involved in DNA repair, transport, transcriptional regulation, energy production, and amino acid metabolism. Again, transposases and bacteriophage-related proteins emerged as helpful for sediment survival. It is of interest that multi-drug resistance mechanisms were highlighted in this organism, while they were not apparent in *D. desulfuricans*. More recently [8], 3,355 transposon-tagged mutants of *S. oneidensis* were screened by microarray. Of these, 1,230 genes demonstrated strong fitness patterns for survival under diverse conditions. An axenic pea rhizosphere model was used to identify colonization mutants by STM in *B. vietnamiensis* [36]. While the majority of mutants identified were in amino acid biosynthesis and general metabolism, some were also identified in gene regulation, transport, and stress.

The very large number of genes identified here will, hopefully, assist in uncovering further mechanisms for bacterial persistence in soil. This could lead to a better assessment of the threat posed by the malicious release of pathogenic bacteria and their ability to persist after release. In the current context, a better understanding of why *B. mallei* is an obligate pathogen may become apparent. Some key examples, highlighted here, of the differences between *B. mallei*, which does not survive in the environment, and *B. thailandensis* are: (i) the lack of motility which was shown to be crucial for soil survival; (ii) the absence of dihydroaeruginoic acid synthetase (BTH_I2367) and pyochelin siderophore (BTH_II2402) production in the obligate pathogen and also its lack of an ortholog to the *Burkholderia* Hep-Hag autotransporter (BuHa) gene (BTH_II0112). BuHA proteins are thought to act as adhesins. Novel means to decontaminate affected areas may also be developed. In a wider view, the role that bacteria play in the cycling of elements and other nutrients in the soil is indispensable to higher life [11]. The

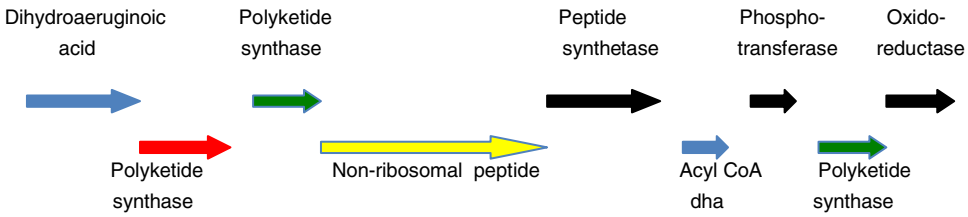

Fig. 3 Predicted operon of genes involved in siderophore synthesis identified by TraDIS. Members of the gene cluster not identified by TraDIS are shown as *black arrows*

promotion of plant growth by bacteria [17] in the rhizosphere is already well known, but will gain scientific interest as the need for food and biomass production increases. Pathogens and agonists of plant pests [2, 46] and diseases [16] are another means to improve plant production. All of these areas and others, such as bioremediation [31], will develop with the increasing knowledge of survival mechanisms of beneficial bacteria and how to promote their growth.

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