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The use of chemical profiling for monitoring metabolic changes in artificial soil slurries caused by horizontal gene transfer

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This study explores the utility of Fourier transform infra-red spectroscopy (FT-IR) as a metabolomic tool to detect changes in water-extractable chemical profile resulting from horizontal gene transfer (HGT) events in artificial soil slurries. A GFP–Km (Green fluorescent protein–kanamycin) cassette tagged HGT recipient *Acinetobacter* strain ADPWH67 with the salicylate hydroxylase gene (*sal*A) disrupted was introduced to slurries containing either sterile or non-sterile soil. The subsequent addition of naked *sal*A DNA allowed the specific monitoring of HGT events by enumerating GFP-expressing colonies on minimal media with salicylate as a sole carbon source. DNA sequencing confirmed that *sal*A was restored in these transformants. Gene transformation frequencies of around 10^{-6} were achieved in the presence of sterile and non-sterile soils. Aqueous extracts of the soil slurries were then analyzed using FT-IR in order to ascertain whether any shifts in chemical profile could be detected. We found that following HGT events FT-IR chemical profiles were clearly altered when analyzed with multivariate statistics. Furthermore, these changes could be explained by differences in key chemical signatures including salicylate as well as other biomolecules found in soils. The slurry extracts were also subjected to GC-MS which confirmed the results of FT-IR analyses. FT-IR was therefore demonstrated to have utility for the rapid screening of metabolomic changes in soils following effective HGT events. In addition, this approach could potentially link specific metabolite changes with corresponding catabolic genes.

KEY WORDS: FT-IR; horizontal gene transfer; *Acinetobacter* ADP1; metabolic; fingerprint.

1. Introduction

Horizontal gene transfer (HGT) is a natural phenomenon in the soil environment and is an important mechanism of gene flow and evolution in bacteria (Ochman *et al*., 2000). In particular, some bacteria such as *Acinetobacter* sp., *Bacillus* sp., *Pseudomonas* sp. and *Haemophilus sp*. are naturally competent and have been shown to take up and integrate foreign DNA from their surrounding soil environment (Lorenz and Wackernagel, 1994). Such events have been shown to generate novel catabolic properties in microbes and there has also been much interest in genetically modified organism (GMO) issues (Herrick *et al*., 1997; Gebhard and Smalla, 1998; Nielsen *et al*., 2000a, b; de Vries *et al*., 2001, 2003; de Vries and Wackernagel, 2002; Kay *et al*., 2002). Despite the use of antibiotic resistance markers coupled with stringent *in vitro* selection methods to actively select transformants, these studies found that stable transformation only occured at a very low frequency (Gebhard and Smalla, 1998; Nielsen *et al*., 2000a, b; de Vries *et al*., 2001; Kay *et al*., 2002). Such experiments provide evidence that HGT can occur under optimised, artificial conditions but reveal relatively little in relation to the possible consequences of such HGT events. It is uncertain, for example, whether the transgenic microbes would survive and multiply such that any new phenotypes which they express might impact on soil ecology. To assess the impact of HGT under less selective conditions requires an environment in which transformants and indigenous bacteria coexist. Such an approach will enable the examination of transformant survival and allow for the asssessment of any global chemical profile changes in soils resulting from HGT.

Traditional chemical profiling analysis techniques such as GC-MS (gas chromatography-mass spectrometry), LC-MS (liquid chromatography-mass spectrometry) and NMR (nuclear magnetic resonance) require relatively complicated sample preparation and using these techniques it is difficult to analyze large numbers of samples in a routine fashion. By contrast, Fourier transform infrared spectroscopy (FT-IR) is a rapid

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technique (typically 8–10 s per sample) that measures vibrations of functional groups within (bio)chemical molecules. Our previous research has shown that FT-IR analysis can achieve much higher throughput by linking the motorised stage of a reflectance thin layer chromatography accessory to a Bruker FT-IR detector; such a technique provides many potential applications. We have reported previously that FT-IR can be used to differentiate soils and earthworm casts (Gwynn-Jones *et al*., 2003; Scullion *et al*., 2003a). The key advantages of this system are that it is rapid, non-invasive, accurate, automated and quantitative and requires minimal sample preparation. We therefore sought to apply this technology to monitor metabolic changes in soil processes arising through HGT.

Earthworms consume soils, plant litters and other organic matter, and excrete wastes in the form of casts, a type of soil aggregate (Davidson *et al*., 2002, 2004). Earthworm casts constitute a large proportion of the organic horizons of most agricultural soils (Davidson *et al*., 2002, 2004) and contain highly active, dense (Clegg *et al*., 1995; Schonholzer *et al*., 2002; Scullion *et al*., 2003b) and diverse microflora (Karsten and Drake, 1995; Furlong *et al*., 2002; Singleton *et al*., 2003). Earthworm casts have been shown to be sites of extensive plasmid conjugation between bacteria (Daane *et al*., 1996, 1997), and may represent specific niches conducive also to HGT. Conversely the highly active microbial community in the earthworm cast environment could present obstacles to HGT by degrading/adsorbing naked DNA or competing with the rare transformants (Nielsen *et al*., 1998). In the present experiments slurries derived from earthworm casts thus provided a challenging natural environment to study the utility of FT-IR for monitoring changes in global chemical profile resulting from a shift in catabolic function caused by HGT.

We have constructed a HGT recipient strain (ADPWH67) with a GFP chromosome tag in which the *sal*A gene has been disrupted by insertion of a DNA cassette containing a kanamycin gene (Km) Thus, the uptake and homologous recombination of exogenous naked DNA containing a functional *sal*A gene from any biological source should restore the capability of ADPWH67 to catabolise salicylic acid. In this study, we constructed an artificial soil environment which can support the growth of both indigenous bacteria and transformants. The data demonstrated that HGT of naked DNA into *Acinetobacter* strain ADPWH67 caused a change in soil metabolome and importantly, such changes could be detected directly in a crude water-extractable chemical profile by FT-IR without the need for bacterial growth and selection *in vitro*.

2. Experimental procedures

2.1. Strains and plasmids

The bacterial strains and plasmids used in this study are shown in table 1.

2.2. Chemicals and medias

Chemicals were obtained from Sigma-Aldrich Co. unless specified otherwise. Luria-Bertani (LB) medium was used to cultivate bacteria unless otherwise noted with selection as appropriate (10 μg/mL kanamycin). *Acinetobacter* transformants were selected on minimal medium (MM) (Na_2HPO_4: 3.0 g, KH_2PO_4: 3.0 g, NH_4Cl: 1.0 g, $MgSO_4 \cdot 7H_2O$: 0.5 g, saturated $CaCl_2$ solution: 10 μL, saturated $FeSO_4$ solution: 10 μL in 1 L water) with 2.5 mM salicylate sodium salt as sole carbon source (SA medium). LB or SA media were solidified

Table 1
The bacterial strains and plasmids used in this study

Strains	Description	Reference or source
Acinetobacter sp. strains		
ADP1 (BD413)	Wild-type, Km^s, SA^+	Juni and Janik, 1969
ADPW67	*sal*A::Km^r; SA^-	Jones *et al*., 2000
ADPWH1	*are*C::GFP + Km^r, Km^r, SA^+, GFP^+	This study
ADPWH67	*salA*::Km^r and *are*C::GFP + Km^r, SA^-, Km^r, GFP^+	This study
E. coli strains		
DH5 α	F^- ϕ80d*lacZ*M15 Δ(*lacZYA-argF*)U169 *deo*R *recA1* *endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ^{-1} *thi-1 gyrA96 relA1*	Gibco
Plasmids		
pUI1637	Km^r; source plasmid for Km^r cassette in pADPW44	Eraso and Kaplan, 1994
pADPW44	2.8 kb Km^r cassette from pUI1637 cloned into *Cla*I site in *sal*A	Jones *et al*., 2000
pADPW21	1.6-kbp *Bam*HI*/*Sac*I fragment containing areC in pUC18	Jones *et al*., 1999
pKOK6	Km^r; source plasmid for Km^r – GFP cassette in pADPW21 G	Kokotek and Lotz, 1989; Jones and Williams, 2003
pADPW21 G	3.6-kbp Km^r–GFP cassette from pKOK6 were cloned into *Bgl*II site in areC. GFP and areC promoter are in the same orientation	This study

with 1.2% agar where appropriate to make LB agar (LBA) and SA agar (SAA) respectively. To estimate transformation frequencies, salicylate-degrading bacteria were plated on SAA with 10 μg/mL kanamycin (SAK) to select for km cassette. Cycloheximide (100 μg/mL) was used in SA media to limit the growth of fungi where appropriate.

2.3. DNA manipulations

Standard molecular techniques were performed as described by Sambrook *et al.* (1989). Total DNA of *Acinetobacter* sp. ADP1 was prepared according to Ausubel *et al.* (2002). Competent cells of *E. coli* DH5α were prepared by a method described previously (Inoue *et al.*, 1990). Plasmids were transformed by electroporation (Gene pulser, Bio-Rad) and maintained in an *E. coli* DH5α host. Plasmid preparation and purification were carried out by using Qiagen plasmid miniprep kit. PCR products were purified using the Qiagen PCR and Gel purification kit. PCR products were sequenced by using the Sanger method (Sanger *et al.*, 1977). DNA sequence analysis was carried out using the GCG and Blast service in NCBI (National Center for Biotechnology Information).

The *sal*A and *sal*AR genes were isolated from ADP1 using PCR. PCR amplifications were carried out in a 50 μL reaction volume which contained 5 μL 10 × reaction buffer (Promega Co.), 200 μM of each deoxynucleoside triphosphate (Invitrogen Co.), 0.5 μM of each primer and 2 units *Taq* DNA polymerase (Promega Co.). A disposable pipette tip was used to transfer a small amount of bacterial colony (0.1–0.25 μL) to the reaction. PCR amplifications were performed with initial denaturation at 95 °C for 5 min, following 35 cycles of 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 1.5 min, and then additional 72 °C for 5 min to finish extension. Amplifications of *sal*A and *sal*AR (partial *sal*A and *sal*R fragment and not covered Km insertion site) were carried out by using primer pair *sal*A_plant_fwd (GTCGACGGAAATGAGTCATGGGTA, forward) or *sal*A_fwdC (TAGATGCTATTTTAGGGAATATTCC) – *sal*A_revH (AACAGGTTGTATTGCTGCTCGC, reverse) and *sal*AR_Bam_fwd (CGCTAAGAATTTGGATCCAGAGTG) – *sal*AR_rev (GACCTGAGTATGCCCGGTAG). PCR products were isolated from a 1% agarose gel, and purified according to manufacturer instruction (QIAquick gel extraction kit, Qiagen Co.). The DNA quantities were estimated by comparing with a standard DNA ladder.

Plasmids used for homologous recombination to construct the recipient *Acinetobacter* strain were linearized by digestion with *Sac*I and incubated with competent ADP1 cells (see below) for 2 h at 28 °C. Transformants were obtained by plating cells on selective media (LBA containing 10 μg/mL kanmaycin).

2.4. Construction of HGT recipient Acinetobacter strain ADPWH67

The construction of the recipient strain (ADPWH67) is shown in figure 1a–c. To allow insertion of a GFP marker gene into the *Acinetobacter* chromosome within the *are* operon (figure 1a) a 3.6-kbp Km^r–GFP cassette from pKOK6 (Jones and Williams, 2003) was cloned into a *Bgl*II site in *are*C to generate pADPW21G (figure 1b). The GFP marker gene and accompanying kanamycin resistance gene were then recombined into the chromosome of ADP1 cells following transformation with pADPW21G (figure 1c). ADP1 does not have any natural fluorescence (figure 1d) and therefore autofluorescent transformant colonies (designated ADPWH1) were selected under UV illumination and shown to stably express GPF in LB medium (figure 1e).

The plasmid pADPW44 containing the *sal*A gene disrupted by insertion of a 2.8 kb Km^r cassette has been described previously (Jones *et al.*, 2000). For construction of ADPWH67, competent cells of ADPWH1 were transformed with pADPW44. Cells were plated on LBA containing 10 μg/mL kanamycin and after 24 h incubation at 28 °C colonies were replica-plated to SAA media to test for ability to utilise salicylate. Colonies unable to grow on SAA but which showed strong GFP expression were further purified and designated ADPWH67 (figure 1f). The DNA insertions in ADPWH1 and ADPWH67 were confirmed by PCR amplification. Primers designed to amplify *sal*A failed to generate a PCR product with ADPWH67 colonies which contained a Km^r cassette disrupting *sal*A. These strains are shown in table 1. Therefore, ADPW67 contains two Km genes, one was inserted in *sal*A and another was in *are*C together with GFP. Therefore, it allows its transformant to express GFP and grow in SAK medium as the *sal*A is restored through transformation. ADPWH67 was used as a recipient strain in all subsequent transformation experiments.

2.5. Bacterial transformation in liquid medium

Competent cells were prepared as before (Palmen *et al.*, 1993, 1994) with slight modifications. Briefly, *Acinetobacter* sp. ADPWH67 was inoculated into 5 mL LB broth in a 25-mL plastic Universal tube and incubated at 28 °C. After shaking (200 rpm) overnight, 250 μL of the culture was diluted into 5 mL fresh LB broth and incubation continued for an additional 2 h at 28 °C. Cells were harvested by centrifugation at 3000 rpm for 5 min and resuspended in 5 mL of medium containing 50% LB broth and 50% SA medium, at a final concentration around 1×10^9 cells/mL. For transformation experiments 1 μg *sal*A DNA fragments were added to 0.5 mL of resuspended competent bacteria in a 25-mL plastic Universal tube. As a

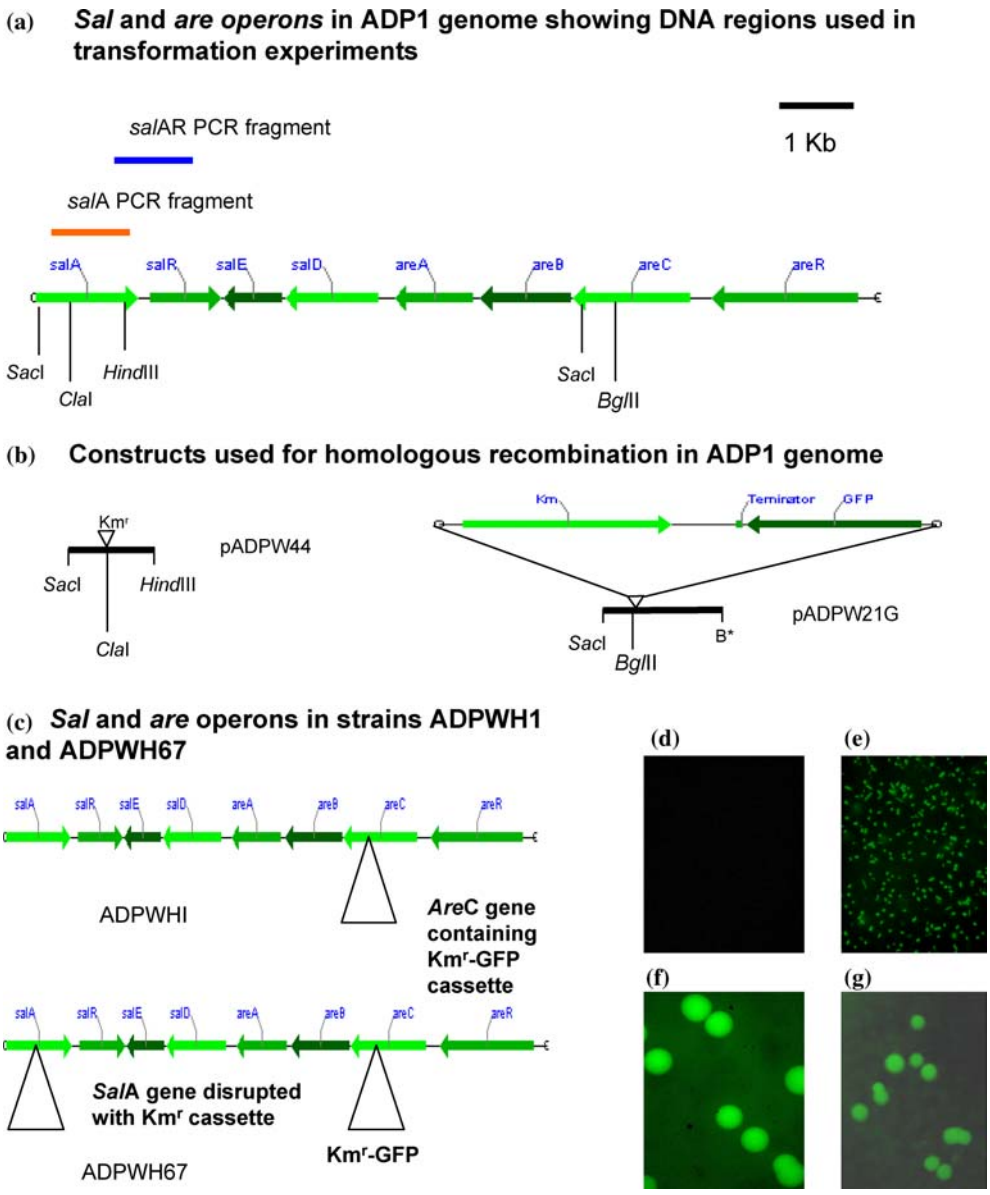

Figure 1. Physical maps of *sal* and *are* operons in ADP1 and construction of the transformation recipient strain ADPWH67. (a)–(c) Illustrate details of the construction of strains ADPWH1 and ADPWH67. (d)–(g) Illustrate *Acinetobacter* sp. ADP1 and its derivatives under UV illumination; (d) ADP1 culture lacking GFP expression from LB medium; (e) ADPWH1 culture showing GFP; (f) ADPWH67 colonies showing GFP expression on LB medium; (g) ADPWH67 transformant colonies after taking up *sal*A gene, showing GFP expression on SAA medium.

transformation control, water or 1 μg of *sal*AR DNA fragments (does not contain a full functional *salA* gene) were added into tubes of competent cells. After mixing the competent cells and naked DNA the tubes were incubated at 28 °C for a further 2 h followed by addition of 50 μg DNAase I to prevent further DNA uptake. Three replicates were performed for each treatment. Ten microlitres of the transformation mixtures were subsequently plated on SAK plates and incubated at 28 °C for 48 h to allow an estimation of transformation frequency. A further aliquot of the transformation mixture was diluted ($1{:}10^5$) and 10 μL plated on LBA for an estimation of total viable cells.

2.6. Transformation in the presence of soil

A standard soil mixture (70% sand, 20% kaoline, 10% peat, w/v) described in previous work (Scullion *et al.*, 2003a) was used in all experiments. Briefly, soil containing populations of the earthworm *Lumbricus terrestris* was maintained in microcosms with a 12.5% moisture content. Sterile soil was produced by autoclaving before use, whilst non-sterile soil was obtained directly from the same microcosms. Non-sterile soil samples consisted mainly of ageing worm casts of *L. terrestris* fed on oat grain which we have shown previously to have enriched source of soil microbial

communities and the total indigenous bacterial population is around 10^9 cells/g casts (Scullion *et al.*, 2003b). For sterile and non-sterile soil experiments, five treatments were carried out and they included a soil only control (treatment A), soil with strain ADPWH67 (treatment B), soil with *Acinetobacter* sp. strain ADPWH1 (treatment C), soil with ADPWH67 and *sal*A DNA (treatment D), and soil with ADPWH67 and *sal*AR (treatment E). Each treatment was carried out in three replicates. Two gram of soils (dry weight) were mixed with 0.5 mL LB and 0.5 mL SA medium in a 25-mL plastic Universal tube to generate a slurry which maintained microbial growth. *Acinetobacter* sp. ADPWH67 or ADPWH1 was then added to the soil slurry at a final concentration of 10^9 cells/g soil. Subsequently, to treatment D and E, 1 μg naked DNA (*sal*A or *sal*AR) from PCR amplifications were added to each tube accordingly. All treatments were then shaken at 50 rpm in a 28 °C incubator. After 2, 24 and 48 h, 0.2 mL of soil slurries from each tube were sampled using a wide orifice pipette tip and 0.2 mL 0.85% NaCl solution added to extract water-soluble soil metabolites, disperse soil particles and release associated micro-organisms. After 10 s of vortexing, 10 μL of supernatant of each sample was taken and plated on SAK to estimate the number of transformants. A diluted ($1{:}10^5$) supernatant (10 μL) was also plated on LBA to estimate total bacterial numbers. For experiments involving non-sterile soil, cycloheximide (100 μg/mL) was added into SAK or LBA to inhibit fungal growth. The rest of the supernatant of each sample was passed through a 0.2 μm filter to remove soil residues and bacteria. The soil extracts were then stored at −80 °C prior to FT-IR and GC-MS analysis.

To estimate transformation frequencies in sterile and non-sterile soils, as described in bacterial transformation in liquid medium, transformation was stopped by adding 50 μg DNAase I to each tube after 2 h incubation. The transformation mixtures were spread on SAK or LBA plates to determine the transformants or total bacteria and estimate transformation frequencies.

2.7. Characterisation of transformants

The identity of putative transformants growing on SAK medium was validated by screening 27 random colonies for the presence of an intact *sal*A gene by PCR. Control colonies were also taken from LB medium. PCR was used to amplify the *sal*A gene using the *sal*A primer pair: *sal*A_plant_fwd and *sal*A_revH. After PCR, the amplified DNA fragments were separated on a 1% agarose gel. The fragments (1277 bp) were then cut from gel, cleaned (QIAquick gel extraction kit, Qiagen Co.) and sequenced using the same primers as in the PCR. The sequence results were compared with *sal*A sequence (AF150928) by standard nucleotide-nucleotide blast analysis (NCIB blast service).

2.8. GFP expression measurement

Colonies of GFP-expressing bacteria growing on plates were observed using a fluorescence stereomicroscope microscope (Leica MZFL III, fitted with a Plan APO 1.6 × lens, and UV lamp). The bacterial colonies expressing GFP were excited by UV light and images were taken using a digital a camera (model Spot RT Colour, Diagnostic Instruments Inc.). Microscopic observations were using an Olympus BX51 epifluorescence microscope equipped with UV for GFP detection. The camera was a Nikon Coolpix 990. The images were processed for display using Microsoft photo editor.

2.9. FT-IR analysis

FT-IR analysis was carried out as described previously (Goodacre *et al.*, 1998, 2000; Ellis *et al.*, 2002; Gwynn-Jones *et al.*, 2003). Fifteen microlitres of soil extract from each sample were placed in individual wells of a 100-well aluminium plate. Before loading into the FT-IR machine, plates loaded with samples were dried in a 50 °C oven for 30 min. The instrument used for FT-IR analysis was a Bruker IFS28 FT-IR spectrometer equipped with a mercury–cadmium–telluride (MCT) detector cooled with liquid N_2. Each sample from the sterile soil experiments was measured three times and the non-sterile soil experiments four times. FT-IR data were collected with Opus software (version 2.1). The scan range of spectra was 4000 to 600 cm^{-1} and resolution was 4 cm^{-1}; each spectrum was represented by 882 points.

2.10. Data analysis

ASCII data were exported from the Opus software and imported into Matlab version 6.1 (The MathWorks, Inc., 24 Prime Par Way, Natick, MA, USA). To minimize baseline shifts (Timmins *et al.*, 1998), spectra were first normalised with the smallest absorbance set to 0 and the highest to + 1 for each spectrum. Smoothed first derivatives of these normalised spectra were then calculated using the Savitzky–Golay algorithm (5-point smoothing). Cluster analysis was used to analyse the FT-IR data (Goodacre *et al.*, 1998; Timmins *et al.*, 1998). Principal components analysis (PCA) (Jolliffe, 1986) was used to reduce the dimensionality of the FT-IR data from 882 to typically 20 PCs (which explained >99% of the total variance). Next, discriminant function analysis (DFA; Manly, 1994) discriminated between groups based on these retained PCs and the *a priori* knowledge of which spectra were machine replicates, a process that does not bias the analysis. Finally, hierarchical cluster analysis (HCA) was used to carry out a similarity measurement by calculating the Euclidean distance between group centres in DFA space (Goodacre *et al.*, 1998, 2000), the similarity matrix was

constructed using Gower's coefficients (S_G), and an agglomerative algorithm used to construct a dendrogam from these similarity measures.

To validate the discrimination performed by PC-DFA, a projection analysis was also employed as detailed in Goodacre *et al.* (2000). Briefly, PC-DFA was carried out as detailed above, randomly chosen test FT-IR spectra, which were not used in the construction of the PC-DFA model, were mathematically projected first into PCA space and then into DFA space generated by the training set (Jarvis and Goodacre, 2004). The key wavenumbers contributing to DFA classifications were recovered by inspection of the PC-DFA loadings plots. These variables were used to re-perform DFA analysis and the data would be considered validated if the re-plotted PC-DFA was the same as in previous PC-DFA analysis.

2.11. GC-MS analysis

Fifty microliters of extract of each soil sample was used for GC-MS measurement. Water was removed *in vacuo* (DNA speed Vac, DNA 120, Savant Co.). Derivatization of dried soil extracts were done by protecting the carbonyl moieties by methoximation using 100 μL of a 20 mg mL^{-1} solution of methoxyamine hydrochloride (Fluka) in pyridine (Fluka) at 30 °C for 90 min. Acidic protons were subsequently derivatized with 100 μL *N*-methyl-*N*-trimethylsilyltrifluoride (MSTFA, M&N) at 37 °C for 30 min. Two μL of the extracts were injected into a GC-MS system consisting of a Focus autosampler (ATAS), an Agilent 6890 N gas chromatograph and a Leco Pegasus III Time-of-Flight detector. Injection temperature was 250 °C, the interface was set to 260 °C and the ion source was adjusted to 230 °C. Helium flow was 1 mL min^{-1}. After 5 min at 80 °C, oven temperature was increased by 60 °C min^{-1} to 330 °C, held at 330 °C for 5 min and cooled to 80 °C. Peak finding was performed using ChromaTof software (Leco). Salicylic acid in samples was quantified using a three-point calibration of standard solutions prepared and analysed accordingly.

3. Results and discussion

3.1. Restoration of salicylic acid catabolism can be used to select horizontal gene transfer events in Acinetobacter spp.

In preliminary transformation experiments in liquid medium using strain ADPWH67 as recipient and *sal*A fragments as donor DNA, transformant colonies were recovered after 48 h growth following the plating of an aliquot of the transformation mixture on SAK medium. All colonies exhibited GFP expression and transformation frequencies were routinely greater than 1×10^{-4}. Twelve fluorescent colonies were picked randomly from SAK plates and all were shown by PCR to contain a DNA fragment of identical size to the intact *sal*A gene present in both in the wild-type ADP1 strain and AD-PWH1 (data not shown). Transformant colonies failed to develop on SAK plates in control experiments utilising the *sal*AR (no intact *salA* gene) DNA fragment as a donor (data not shown).

3.2. Acinetobacter exhibits relatively high transformation rates by naked DNA even in the presence of non-sterile soil

A series of transformation experiments were established to provide material for FT-IR analysis and to determine the effect of soil on the transformation frequency. A GFP and Km tagged strain of *Acinetobacter* ADPWH67 allowed simple discrimination between endogenous bacteria and transformed colonies in treatments containing soil. Five treatments were performed in slurries containing either sterile or non-sterile soil which harboured with active indigenous microbial populations. Treatment D was the actual transformation treatment in which it was expected that the recipient ADPWH67 could gain the capacity to catabolize salicylate following acquisition of a naked *sal*A DNA added into the soil/medium mixture. Treatment E represented a further transformation control using a DNA fragment of the *sal* operon which does not cover the disruption site (*Cla*I) of *sal*A and thus cannot regenerate a functional *sal*A gene after homologous recombination.

In sterile soil experiments, colonies did not develop on SAK plates in control treatments A, B and E. By contrast, colonies were recovered on SAK plates from positive control treatment C and transformation treatment D and all of them exhibited strong GFP expression. In the non-sterile soil experiments, a few colonies developed on SAK plates in treatments A, B and E. However, these colonies did not show GFP expression and were considered to be indigenous bacteria able to utilise salicylate. In treatment D, both GFP-expressing and non-fluorescent colonies were recovered but only those showing GFP were counted as genuine transformants and chosen for further analysis. PCR amplifications were performed by randomly selecting GFP positive colonies growing on SAK medium from three replicates of treatment D (both sterile and non-sterile soil) and results were shown to contain a *salA* gene identical in size to the wild-type gene in ADP1 and ADPWH1, which was absent in the recipient bacteria ADPWH67 (data not shown). Sequence analysis of DNA amplified from 12 putative transformants using the *sal*A_fwdC and salA_revH primers confirmed the presence of a full-length *sal*A gene (data not shown).

A single bacterial species can dominate within the microbial communities of earthworm casts and levels as high as 28% have been reported (Furlong *et al.*, 2002).

Assuming the total bacterial community can be as high as 10^{10} cells per gram of cast (Clegg *et al*., 1995; Schonholzer *et al*. 2002; Scullion *et al*. 2003b), it is not unrealistic to add 10^9 recipient cells per gram slurries to mimic natural conditions. All transformation frequencies were estimated on the basis of 2 h mixing of naked DNA with LB or sterile/non-sterile soils as described in Materials and methods. Transformation frequencies were reduced by two orders of magnitude to 2.76×10^{-6} and 9.02×10^{-6} in sterile and non-sterile soil, compared with 1.20×10^{-4} in optimal transformation conditions (LB liquid gene transfer). This observation suggests that *sal*A DNA fragments survived at least 2 h in non-sterile soil and escaped degradation.

In treatments using non-sterile soils the total bacterial populations were greater than 10^9 cells per gram soil and thus any *Acinetobacter* transformants had to compete with a large number of indigenous bacteria also able to degrade salicylate. Thus it was not surprising that in the non-sterile soil experiment, *Acinetobacter* transformants constituted only 26% of all colonies growing on SAK plates and were not yet the dominant salicylate degrading species 2 h after transformation. Thereafter the population of *Acinetobacter* transformants in treatment D increased from 1.01 (± 0.52) $\times 10^4$ in 2 h to 3.35 (± 0.68) $\times 10^5$ in 24 h and reached in excess of 10^8 cells per wet gram soil in 48 h treatments. The number of transformants recovered was similar in the presence of both sterile and non-sterile soil. Since DNase was added after 2 h incubation, we presume that most of the subsequent increases are due to outgrowth of existing rather than new transformants. The physiological state of bacteria, availability of naked DNA source, selection, competition and environmental impact of bacterial transformants are all known to affect the success of HGT. The present data suggest that *Acinetobacter* inocula were still able to exhibit high competence levels, at least in the presence of the microflora found in worm casts. Furthermore, the new catabolic function (salicylate degradation) acquired by transformants through HGT allowed the recipients to be highly competitive when salicylate was added.

3.3. Multivariate cluster analysis of FT-IR spectra can detect alterations of chemical profile in soil water associated with transformation treatment

3.3.1. Sterile soil slurries

Figure 2 shows the PC-DFA cluster analysis of all treatments of sterile soils after 2 and 24 h. It is clear that as the medium was utilised, the chemical profile of the soils changed, and this change was different for the various treatments. A major difference was seen in the first discriminant function (DF) axis between the clustering of aqueous extracts of Treatment D and Treatments E and B which all contain strain ADPWH67, with 24-h samples showing the most separation. By contrast,

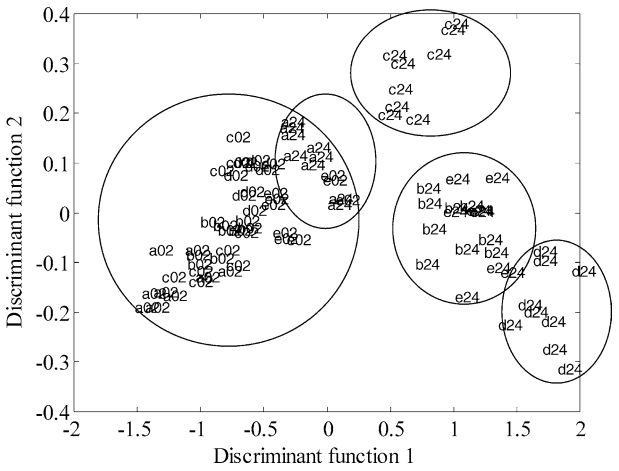

Figure 2. DFA analysis of FT-IR chemical profiles of extracts of sterile soil slurries. Samples from treatments A, B, C, D and E in sterile soil experiments are represented by letters a, b, c, d and e. The numbers represent the minimum incubation time (02–2 h, 24–24 h) after adding DNA into soils. Each treatment had three replicates and each sample was measured three times.

treatment A (the soil control) showed little change in the chemical profiles. The treatments containing predominantly bacteria (ADPWH67) unable to metabolise salicylate (Treatments B and E) were difficult to separate in both of these data visualisations (figure 2 and supplement figure A).

GC-MS profiling of soil extracts confirmed that the transformation events in the soil slurries had indeed led to a change in levels of soil chemical profiles with a specific reduction in SA concentration (data not shown). In treatment C (positive control) the majority of the SA had disappeared within 24 h. In the functional transformation (treatment D) SA was depleted from the medium by 48 h but was still detectable in some replicate samples at 24 h as evidenced by the high standard deviation in these samples. SA levels were unchanged in other treatments.

3.3.2. Non-sterile soil slurries

Plating on SAK media had previously indicated that indigenous bacteria existed in non-sterile soils that were able to degrade salicylate. PC-DFA of the FT-IR spectra showed that the chemical profiles in samples containing non-sterile soil cluster strongly on the basis of the hours of incubation following treatment (figure 3a). GC-MS analysis indicated that nucleic acids, most water-soluble lipids, carbohydrates and other organic compounds had been significantly reduced during the time course of these treatments, and also indicated that SA was below detection levels after 48 h incubation in all treatments (data not shown). The results indicate that indigenous bacterial communities were highly active and rapidly consumed the nutrients in non-sterile soils. Data from samples incubated for 24 h were reanalyzed in isolation by PC-DFA (figure 3b). The data of 10 samples were used as training data and they were classified

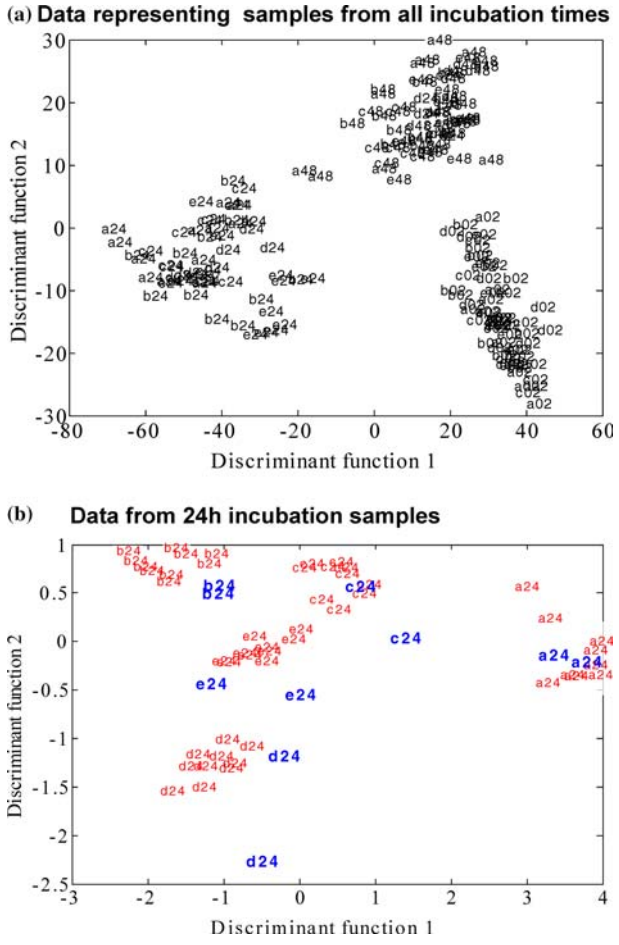

Figure 3. DFA analysis of FT-IR chemical profiles of extracts of non-sterile soil slurries. Samples from treatments A, B, C, D and E in non-sterile soil experiments are represented by letters a, b, c, d and e. The numbers represent the minimum incubation time (02–2 h, 24–24 h and 48–48 h) after adding DNA into soil slurries. Each treatment had three replicates and each sample was measured four times. (a) Chemical profiles in samples cluster strongly on the basis of the hours of incubation following treatment indicate bacterial communities were highly active and consumed the nutrients in soils. (b) PC-DFA projection analysis for the samples of 24 h treatments. For each treatment, the data of ten samples used as training data are shown in normal font. The data of two samples used as testing data to validate the classification are shown in bold.

into five groups. The data of two samples were used as testing data to validate the classification, as detailed in Materials and methods. The results shows that the two test data generally grouped into five separate clusters, confirming that the cluster analysis was valid. To clearly visualize the relative similarity and relationship between these five treatments, a dendrogram constructed from DFA space is also shown (supplement figure B). The key wavenumbers that contributed to the soil PC-DFA classifications were recovered from both sterile (figure 2) and non-sterile (24-h treatment, figure 3b) experiments (table 2) by inspection of the PC-DFA loadings plots (data not shown). Table 2 shows that, in both sterile and non-sterile soil experiments, the variations in salicylate

Table 2
The key wavenumbers contributed for the DFA classification in soil gene transfer experiments and their putative assignments

Sterile soils wavenumbers (cm^{-1})	Putative assignment	Non-sterile soils (24 h treatments) wavenumbers (cm^{-1})	Putative assignment
1701–1708	Salicylic acid, aromatic combination bands (Coates, 2000)	2916–2924	C–H stretching of $>CH_2$ in fatty acids (Naumann, 2001; Maquelin *et al.*, 2002)
1712–1716	$>C=O$ in nucleic acids (Naumann, 2001; Maquelin *et al.*, 2002)	1712–1716	$>C=O$ in nucleic acids (Naumann, 2001; Maquelin *et al.*, 2002)
1697	Amide I bands from antiparallel pleated sheets and β-turns of protein (Naumann, 2001; Maquelin *et al.*, 2002)	1701–1709	Salicylic acid, aromatic combination bands (Coates, 2000)
1654	Amide I of α-helical structures (Naumann, 2001; Maquelin *et al.*, 2002)	1462–1466	C–H deformation of $>CH_2$ (Naumann, 2001; Maquelin *et al.*, 2002)
1141–1164	Salicylic acid (Coates, 2000)	1157–1164	Salicylic acid (Coates, 2000)
1168–1191	C–O–C/C–O–P stretching in carbohydrates (Naumann, 2001; Maquelin *et al.*, 2002)	1168–1188	C–O–C/C–O–P stretching in carbohydrates (Naumann, 2001; Maquelin *et al.*, 2002)
1083 cm^{-1}	PO_2^- in nucleic acids (Naumann, 2001; Maquelin *et al.*, 2002)	980–987	C–O–C/C–O–P stretching in carbohydrates (Naumann, 2001; Maquelin *et al.*, 2002)

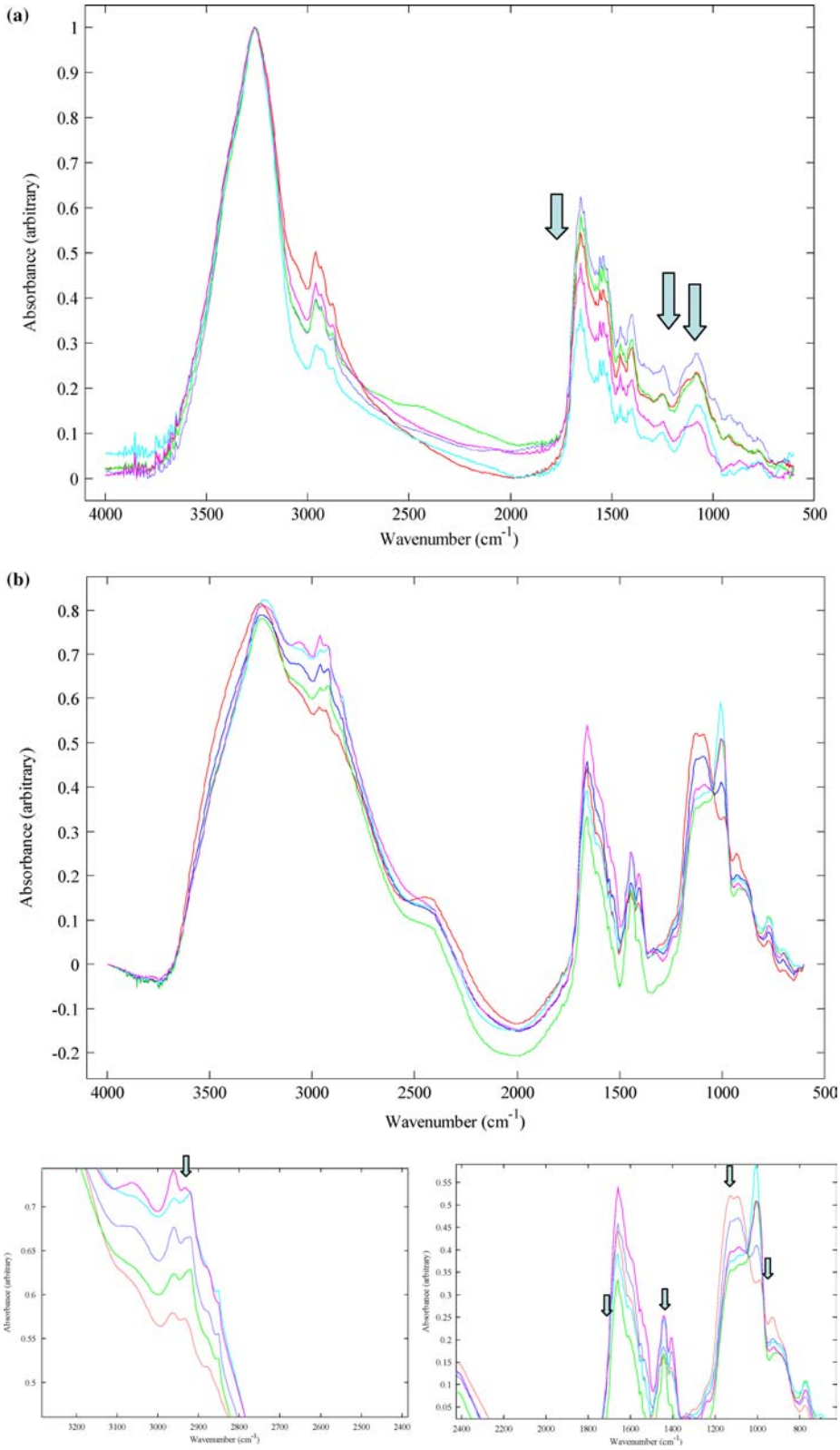

Figure 4. Comparison of spectra of five treatments of both sterile and non-sterile soil experiments. The red, blue, green, cyan and magenta represent treatments A, B, C, D and E respectively. Each spectrum is averaged all replicates. Arrows show the wavenumbers that contributed to the classification as highlighted in table 2. (a) Sterile soil experiment comparison. (b) Non-sterile soil experiment comparison. Two lower panels provide detail of specific regions of the FT-IR spectra.

and nucleic acids contribute to the grouping patterns of FT-IR spectra. Figure 4a and b show the comparison of the spectra of five treatments in both experiments. Salicylate changes were wholly consistent with the results of GC-MS analysis. Fatty acids recovered in non-sterile soils experiments (24 h treatment) indicate that bacterial populations changed in the different treatments and this was consistent with bacterial counting observations. The FT-IR analyses (putative assignments in table 2) support the changes detected in nucleic acids, lipids, carbohydrates and salicylic acid by GC-MS analysis. The information derived from FT-IR analysis, is thus consistent with the observations that bacterial populations had changed in treatment D following gene transformation and build up of salicylate degrading bacteria and provided clues of the impact of HGT in this complex worm cast system.

In summary, we have demonstrated that *Acinetobacter* sp. can be readily transformed to restore the function of *sal*A in liquid and artificial soil slurry systems. In non-sterile soil experiments, we constructed an environment which allowed *Acinetobacter* transformants and indigenous bacteria to coexist to compete for resources. The transformants gradually developed to be dominant within 48 h and changed the chemical profiles of the soils when conditions were favourable. We have also shown that the consequence of gene transformation in soil slurries can be monitored by FT-IR. The data suggested that soil chemical profiles changed as a result of effective HGT events and that bacteria obtain new catabolic functions in a short time. In contrast, soil chemical profiles remained virtually unchanged when HGT events were not functional. GC-MS analyses supported the results of FT-IR analysis in both sterile and non-sterile soil experiments. We conclude that although the FT-IR approach is not directly quantitative for transformation frequency, nevertheless it does provide a rapid and specific measure of functional changes in the soil slurries This is a high throughput process (typical analysis time is 8–10 s per sample) and generates highly reproducible data allowing clear discrimination between the different treatments. This type of approach does not demand an ability to extract and culture individual microbes *in vitro*. More importantly, the FT-IR data applied to metabolic studies can elucidate the chemical changes in complex systems and indicate possible changes in catabolic gene functions. It potentially provides a bridge linking the metabolites and gene functions. Thus, these data reflect both changes at the microbial community level as well as specific alterations in metabolic activity of the transformed individuals. Therefore, rather than simply trying to measure the frequency of HGT such techniques may make it possible to assess the impact of environmental disturbance on the soil bacterial community as a whole.

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References

Ausubel, F.M., Brent, R., Kingston, R.E., *et al*. (eds). (2002). Current Protocols in Molecular Biology, Vol. 1. John Wiley & Sons Inc., New York, NY.

Clegg, C.D., Anderson, J.M., Lappinscott, H.M., Vanelsas, J.D. and Jolly, J.M. (1995). Interaction of a genetically-modified *Pseudomonas fluorescens* with the soil-feeding earthworm *Octolasion cyaneum* (Lumbricidae). *Soil Biol. Biochem.* **27**, 1423–1429.

Coates, J. (2000). *Encyclopedia of Analytical Chemistry*. John Wiley & Sons Ltd., Chichester.

Daane, L.L., Molina, J.A.E., Berry, E.C. and Sadowsky, M.J. (1996). Influence of earthworm activity on gene transfer from *Pseudomonas fluorescens* to indigenous soil bacteria. *Appl. Environ. Microbiol.* **62**, 515–521.

Daane, L.L., Molina, J.A.E. and Sadowsky, M.J. (1997). Plasmid transfer between spatially separated donor and recipient bacteria in earthworm-containing soil microcosms. *Appl. Environ. Microbiol.* **63**, 679–686.

Davidson, D.A., Bruneau, P.M.C., Grieve, I.C. and Young, I.M. (2002). Impacts of fauna on an upland grassland soil as determined by micromorphological analysis. *Appl. Soil Ecol.* **20**, 133–143.

Davidson, D.A., Bruneau, P.M.C., Grieve, I.C. and Wilson, C.A. (2004). Micromorphological assessment of the effect of liming on faunal excrement in an upland grassland soil. *Appl. Soil Ecol.* **26**, 169–177.

de Vries, J., Meier, P. and Wackernagel, W. (2001). The natural transformation of the soil bacteria *Pseudomonas stutzeri* and *Acinetobacter* sp. by transgenic plant DNA strictly depends on homologous sequences in the recipient cells. *FEMS Microbiol. Lett.* **195**, 211–215.

de Vries, J., Heine, M., Harms, K. and Wackernagel, W. (2003). Spread of recombinant DNA by roots and pollen of transgenic potato plants, identified by highly specific biomonitoring using natural transformation of an *Acinetobacter* sp. *Appl. Environ. Microbiol.* **69**, 4455–4462.

de Vries, J. and Wackernagel, W. (2002). Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. *Proc. Natl. Acad. Sci. USA* **99**, 2094–2099.

Ellis, D.I., Broadhurst, D., Kell, D.B., Rowland, J.J. and Goodacre, R. (2002). Rapid and quantitative detection of the microbial spoilage of meat by Fourier transform infrared spectroscopy and machine learning. *Appl. Environ. Microbiol.* **68**, 2822–2828.

Eraso, J.M. and Kaplan, S. (1994). Prra, a putative response regulator involved in oxygen regulation of photosynthesis gene-expression in *Rhodobacter sphaeroides*. *J. Bacteriol.* **176**, 32–43.

Furlong, M.A., Singleton, D.R., Coleman, D.C. and Whitman, W.B. (2002). Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. *Appl. Environ. Microbiol.* **68**, 1265–1279.

Gebhard, F. and Smalla, K. (1998). Transformation of *Acinetobacter* sp. strain BD413 by transgenic sugar beet DNA. *Appl. Environ. Microbiol.* **64**, 1550–1554.

Goodacre, R., Timmins, E.M., Burton, R., *et al.* (1998). Rapid identification of urinary tract infection bacteria using hyperspectral whole-organism fingerprinting and artificial neural networks. *Microbiology-UK* **144**, 1157–1170.

Goodacre, R., Shann, B., Gilbert, R.J., *et al.* (2000). Detection of the dipicolinic acid biomarker in *Bacillus* spores using Curie-point pyrolysis mass spectrometry and Fourier transform infrared spectroscopy. *Anal. Chem.* **72**, 119–127.

Gwynn-Jones, D., Huang, W.E., Easton, G., Goodacre, R. and Scullion, J. (2003). UV-B radiation induced changes in litter quality affects earthworm growth and cast characteristics as determined by metabolic fingerprinting. *Pedobiologia* **47**, 784–787.

Herrick, J.B., StuartKeil, K.G., Ghiorse, W.C. and Madsen, E.L. (1997). Natural horizontal transfer of a naphthalene dioxygenase gene between bacteria native to a coal tar-contaminated field site. *Appl. Environ. Microbiol.* **63**, 2330–2337.

Inoue, H., Nojima, H. and Okayama, H. (1990). High efficiency transformation of *E. coli* with plasmids. *Gene* **96**, 23–28.

Jarvis, R.M. and Goodacre, R. (2004). Discrimniation of bacteria using surface-enhanced Raman spectroscopy. *Anal. Chem.* **76**, 40–47.

Jolliffe, I.T. (1986). *Principal Component Analysis*. Springer, New York.

Jones, R.M., Collier, L.S., Neidle, E.L. and Williams, P.A. (1999). *areABC* genes determine the catabolism of aryl esters in *Acinetobacter* sp. strain ADP1. *J. Bacteriol.* **181**, 4568–4575.

Jones, R.M., Pagmantidis, V. and Williams, P.A. (2000). *sal* genes determining the catabolism of salicylate esters are part of a supraoperonic cluster of catabolic genes in *Acinetobacter* sp. strain ADP1. *J. Bacteriol.* **182**, 2018–2025.

Jones, R.M. and Williams, P.A. (2003). Mutational analysis of the critical bases involved in activation of the AreR-regulated sigma(54)-dependent promoter in *Acinetobacter* sp. strain ADP1. *Appl. Environ. Microbiol.* **69**, 5627–5635.

Juni, E. and Janik, A. (1969). Transformation of *Acinetobacter calcoaceticus* (*Bacterium anitratum*). *J. Bacteriol.* **98**, 281–288.

Karsten, G.R. and Drake, H.L. (1995). Comparative-assessment of the aerobic and anaerobic microfloras of earthworm guts and forest soils. *Appl. Environ. Microbiol.* **61**, 1039–1044.

Kay, E., Vogel, T.M., Bertolla, F., Nalin, R. and Simonet, P. (2002). In situ transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. *Appl. Environ. Microbiol.* **68**, 3345–3351.

Kokotek, W. and Lotz, W. (1989). Construction of a *LacZ*–kanamycin-resistance cassette, useful for site-directed mutagenesis and as a promoter probe. *Gene* **84**, 467–471.

Lorenz, M.G. and Wackernagel, W. (1994). Bacterial gene-transfer by natural genetic-transformation in the environment. *Microbiol. Rev.* **58**, 563–602.

Manly, B.F.J. (1994). *Multivariate Statistical Methods: a Primer*. Chapman & Hall, London.

Maquelin, K., Kirschner, C., Choo-Smith, L.P., *et al.* (2002). Identification of medically relevant microorganisms by vibrational spectroscopy. *J. Microbiol. Meth.* **51**, 255–271.

Naumann, D. (2001). FT-infrared and FT-Raman spectroscopy in biomedical research. *Appl. Spectr. Rev.* **36**, 239–298.

Nielsen, K.M., Bones, A.M., Smalla, K. and van Elsas, J.D. (1998). Horizontal gene transfer from transgenic plants to terrestrial bacteria – a rare event?. *FEMS Microbiol. Rev.* **22**, 79–103.

Nielsen, K.M., Smalla, K. and van Elsas, J.D. (2000a). Natural transformation of *Acinetobacter* sp. strain BD413 with cell lysates of *Acinetobacter* sp., *Pseudomonas fluorescens*, and *Burkholderia cepacia* in soil microcosms. *Appl. Environ. Microbiol.* **66**, 206–212.

Nielsen, K.M., van Elsas, J.D. and Smalla, K. (2000b). Transformation of *Acinetobacter* sp. strain BD413 (pFG4 Delta nptII) with transgenic plant DNA in soil microcosms and effects of kanamycin on selection of transformants. *Appl. Environ. Microbiol.* **66**, 1237–1242.

Ochman, H., Lawrence, J.G. and Groisman, E.A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**, 299–304.

Palmen, R., Vosman, B., Buijsman, P., Breek, C.K.D. and Hellingwerf, K.J. (1993). Physiological characterization of natural transformation in *Acinetobacter calcoaceticus*. *J. Gen. Microbiol.* **139**, 295–305.

Palmen, R., Buijsman, P. and Hellingwerf, K.J. (1994). Physiological Regulation of competence induction for natural transformation in *Acinetobacter calcoaceticus*. *Arch. Microbiol.* **162**, 344–351.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. (second ed.). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Schonholzer, F., Hahn, D., Zarda, B. and Zeyer, J. (2002). Automated image analysis and in situ hybridization as tools to study bacterial populations in food resources, gut and cast of *Lumbricus terrestris* L.. *J. Microbiol. Meth.* **48**, 53–68.

Scullion, J., Elliot, G.N., Huang, W.E., *et al.* (2003a). Use of earthworm casts to validate FT-IR spectroscopy as a 'sentinel' technology for high-thoughput monitoring of global changes in microbial ecology. *Pedobiologia* **47**, 440–446.

Scullion, J., Goodacre, R., Elliott, G., *et al.* (2003b). Food quality and microbial succession in ageing earthworm casts: standard microbial indices and metabolic fingerprinting. *Pedobiologia* **47**, 888–894.

Singleton, D.R., Hendrix, P.F., Coleman, D.C. and Whitman, W.B. (2003). Identification of uncultured bacteria tightly associated with the intestine of the earthworm *Lumbricus rubellus* (Lumbricidae; Oligochaeta). *Soil Biol. Biochem.* **35**, 1547–1555.

Timmins, E.M., Howell, S.A., Alsberg, B.K., Noble, W.C. and Goodacre, R. (1998). Rapid differentiation of closely related *Candida* species and strains by pyrolysis mass spectrometry and Fourier transform-infrared spectroscopy. *J. Clin. Microbiol.* **36**, 367–374.