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Low-Intensity Microwave Irradiation Does Not Substantially Alter Gene Expression in Late Larval and Adult *Caenorhabditis elegans*

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Reports that low-intensity microwave radiation induces heat-shock reporter gene expression in the nematode, *Caenorhabditis elegans*, have recently been reinterpreted as a subtle thermal effect caused by slight heating. This study used a microwave exposure system (1.0 GHz, 0.5 W power input; SAR 0.9–3 mW kg^{-1} for 6-well plates) that minimises temperature differentials between sham and exposed conditions ($\leq$0.1 °C). Parallel measurement and simulation studies of SAR distribution within this exposure system are presented. We compared five Affymetrix gene arrays of pooled triplicate RNA populations from sham-exposed L4/adult worms against five gene arrays of pooled RNA from microwave-exposed worms (taken from the same source population in each run). No genes showed consistent expression changes across all five comparisons, and all expression changes appeared modest after normalisation ($\leq$40% up- or down-regulated). The number of statistically significant differences in gene expression (846) was less than the false-positive rate expected by chance (1131). We conclude that the pattern of gene expression in L4/adult *C. elegans* is substantially unaffected by low-intensity microwave radiation; the minor changes observed in this study could well be false positives. As a positive control, we compared RNA samples from N2 worms subjected to a mild heat-shock treatment (30 °C) against controls at 26 °C (two gene arrays per condition). As expected, heat-shock genes are strongly up-regulated at 30 °C, particularly an *hsp*-70 family member (C12C8.1) and *hsp*-16.2. Under these heat-shock conditions, we confirmed that an *hsp*-16.2::GFP transgene was strongly up-regulated, whereas two non-heat-inducible transgenes (*daf*-16::GFP; *cyp*-34A9::GFP) showed little change in expression. Bioelectromagnetics 30:602–612, 2009.

Key words: microwave radiation; gene expression; gene arrays; *Caenorhabditis elegans*

INTRODUCTION

In a previous report, we suggested that low-intensity microwave fields (similar to those generated by mobile phones) could induce a non-thermal heat-shock response in the nematode *Caenorhabditis elegans* [de Pomerai et al., 2000]. This effect has since been reinterpreted as a subtle thermal artefact caused by small temperature disparities ($\leq$0.2 °C) between exposed and sham conditions [Dawe et al., 2006]. A modified TEM exposure cell was used to reduce this temperature differential substantially (to $\leq$0.1 °C), but this also abolished any detectable heat-shock response. Moreover, a quantitatively similar increase in heat-shock reporter gene expression could be induced by a temperature rise of 0.2 °C in the absence of any applied microwave field

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[Dawe et al., 2006]. Similarly, slight heating could explain why mutant phenotype prevalence is increased by microwave exposure in several temperature-sensitive *C. elegans* mutants grown at an intermediate temperature [Gul-Guven et al., 2006].

Although the heat-shock response is useful as a general indicator of cellular stress [see, e.g. de Pomerai, 1996], it goes without saying that the expression of many other genes and signalling pathways could be affected by microwave exposure. Several published gene-array studies document significant changes in the expression of a subset of genes following microwave irradiation [e.g. Belyaev et al., 2006; Remondini et al., 2006; Zhao et al., 2007a,b], whereas other gene-array studies have reported no significant alterations [Gurisik et al., 2006; Qutob et al., 2006; Whitehead et al., 2006]. These published studies in all cases use vertebrate cell cultures exposed to simulated GSM fields (at 0.9 or 1.8 GHz) at a moderate SAR approaching 2.0 W kg^{-1}. The present study utilised the model nematode, *C. elegans*, which was exposed to continuous-wave (CW) 1.0 GHz fields for 2.5 h at a much lower SAR of 0.9–3 mW kg^{-1} [cf. de Pomerai et al., 2000; Dawe et al., 2006]. Pooled RNA samples and multiple Affymetrix chips (5× sham vs. 5× exposed) were used to look for consistent microwave-induced changes in the pattern of gene expression.

MATERIALS AND METHODS

The wild-type (N2) strain of *C. elegans* was originally obtained from the MRC Laboratory of Molecular Biology at Cambridge (UK), as was the P90C *lac*-deleted strain of *Escherichia coli* used as a food source. *C. elegans* strain CL2070 (*hsp*-16.2::GFP) was generously donated by Chris Link (University of Colorado, Boulder, CO), strain TJ356 (*daf*-16::GFP) was from the *Caenorhabditis* Genetics Centre (University of Minnesota, St Louis, MO) and strain BC20306 (*cyp*-34A9) was supplied by the GFP fusion-gene project (headed by David Baillie, Simon Fraser University, Vancouver, Canada). Trizol was obtained from Invitrogen (Paisley, UK). The commercially available Affymetrix *C. elegans* Genome Array (Affymetrix, Santa Clara, CA) was used for all experiments.

Worm Culture

N2 worms were cultured at 15 °C on large 14 cm Petri dishes containing nematode growth medium (NGM) agar overlaid with a lawn of food bacteria (*E. coli* strain P90C), as described previously [Sulston and Hodgkin, 1988; Dawe et al., 2006]. Worms were then synchronised by egg isolation using bleach [Sulston and Hodgkin, 1988] and the L1 offspring were filtered using 5 μm nylon filters [Mutwakil et al., 1997]. These synchronised cultures were grown up to the L4 stage before exposure. L4 worms were washed off the plates using ice-cold K medium (53 mM NaCl, 32 mM KCl) [Williams and Dusenbery, 1990], filtered using 5 μm nylon filters to remove excess bacteria and then dispensed carefully using a magnetic stirrer into two 6-well plates destined for immediate microwave or sham exposure, respectively. The sample volume in each well was always 1.0 ml, as previously used for dosimetry and temperature measurements (see below).

Microwave and Mild Heat Exposure

One group of worms was exposed to the microwave field (CW; 1.0 GHz; 0.9–3 mW kg^{-1}) for 2.5 h at 26 °C in the modified silver-plated TEM cell described in Dawe et al. [2006], whereas the other group was sham-exposed (no field) for the same length of time at the same temperature in an unmodified copper TEM cell. The temperature difference between exposed samples in the silver-plated cell and sham samples in the copper cell has previously been measured at $\leq$0.1 °C [Dawe et al., 2006], thus minimising the contribution of thermal artefacts to this study. As a positive control, we also compared gene-expression profiles between N2 worms at 30 °C (mild heat shock) and at 26 °C (control), using a shorter exposure time of 1.5 h (see text for explanation).

Dosimetry and SAR Modelling

Using 24-well plates containing 1.0 ml of K medium per well, the specific absorption rate (SAR) was measured using an isotropic IndexSAR IXP-010 E-field probe (details at www.indexsar.com/dosimetric-probe.htm) with a 900 MHz CW signal at 1.0 W input power. Output power from the cell was also monitored using a calibrated power sensor, and the results were normalised to 1.0 W into a 50 Ω load. The single-axis probe has an outer diameter of 1.5 mm and is minimally perturbing to the fields being measured; it was calibrated for SAR in K medium, with the dipole sensor arranged at an angle of 54.7° to the axis (so that the isotropic field is given by the sum of the output voltage measured at three positions 120° apart, rotated on the probe axis). The probe was positioned so as to dip into the K medium with its tip midway between the meniscus and the well floor, using a precision linear slide. This was repeated for each of the 24-well positions. The isotropy of this probe is better than 1 dB. These measurements were conducted at the UK National Physical Laboratory (NPL) as part of a complete calibration of the Nottingham TEM cell used here and previously.

Numerical computer simulations were also used to model the exposure of 1.0 ml of K medium per well in both 6- and 24-well plate formats placed inside this TEM cell, based on the 1.0 GHz field and 0.5 W power input actually used in our experiments. The computations were performed using a commercial software tool XFDTD (Remcom, State College, PA)-based finite difference in time domain (FDTD) method. The geometry of the TEM cell, multiwell plates and K medium (1.0 ml) within each well in the simulation model was as close as possible to the experimental setup and was discretised with a minimum discrimination step of 0.5 mm using a variable grid. The dielectric constant of K medium in simulations was 78.2 (measured using an HP 85070C Dielectric Probe kit) while conductivity was 1.16 S/m; SAR was computed for a mass density of $1\,g\,cm^{-3}$. The greyscale squares in Figure 2B,C show average SAR per $0.5\,mm^3$ voxel in the basal layer of K medium, centred 0.25 mm above the floor of each well. For direct comparison with probe measurements (above), we also modelled the SAR distribution in a layer of medium midway between the well floor and meniscus in the 24-well format (Figure 2A). SAR modelling for other layers of liquid within the sample is available as a Powerpoint presentation (shown in Supplementary Material 1).

RNA Extraction

Aliquots of L4 worms were either sham-exposed (control; no field) in a copper TEM cell, or exposed to 1.0 GHz, 0.5 W for 2.5 h at 26 °C in a silver-plated TEM cell [Dawe et al., 2006]. In a separate experiment, batches of L4 worms were incubated (again in 6-well plates) for 1.5 h at either 26 °C (cf. sham controls) or 30 °C (mild heat shock). Post-exposure, the worms were quickly removed from the 6-well dishes using glass pipettes, pelleted by centrifugation (3000*g* for 3 min) and then dropped in small concentrated pellets (again using a glass pipette) into liquid nitrogen. These pellets were then crushed using a precooled mortar and pestle (−80 °C) in the presence of 2 ml of Trizol (Invitrogen). The worm–Trizol slurry was transferred by spatula into a 50 ml tube, left to defrost at room temperature with regular agitation, and then transferred to 2×1.5 ml microcentrifuge tubes. A standard Trizol RNA extraction was then performed according to the manufacturer's instructions. All microcentrifuge tubes, the spatula and mortar and pestle were autoclaved and pretreated with RNaseZap (Ambion, Huntingtdon, UK) and diethyl pyrocarbonate (DEPC)-treated water prior to use. RNA samples from three exposure runs (performed on different days) were combined for hybridisation onto microarrays. Thus the results below are derived from 5 sets of sham gene arrays pooled from 15 sham exposures, compared against 5 sets of exposed gene arrays pooled from 15 microwave exposures. RNA was transported on dry ice and stored at −80 °C. Only two gene arrays and two replica runs per test condition were used for the 1.5 h positive controls, where mild heat shock at 30 °C was compared against 26 °C shams. Air temperatures were monitored continuously throughout these positive control experiments using Gemini TinyTalk™ temperature loggers (RS Components, Corby, UK) with a thermosensor sensitivity of ± 0.5 °C, reporting every 20 s. Temperature recordings for these runs are available as Supplementary Material 2; the average temperatures are very close to 25.5 and 30 °C, respectively, but readings fluctuate as expected across a range of 1.0 °C (mean ± 0.5 °C). To minimise the time taken for temperature acclimation, the 6-well plates containing 1.0 ml per well of K medium were prewarmed for several hours in the respective 26 and 30 °C incubators. These plates were removed very briefly for addition of worm aliquots (as above) and were then returned to the same incubator.

RNA Labelling and Hybridisation to Affymetrix Gene Chips

RNA quality was analysed with the Agilent 2100 Bioanalyser (Agilent Technologies, Geneva, Switzerland) using the RNA 6000 nano kit. All 14 RNA samples were of sufficient quality for gene array analysis, with 28S:18S rRNA ratios of between 1.8 and 2.7. Approximately 5 µg of total RNA from each sample was used to produce cDNA using the GeneChip® One-cycle cDNA synthesis kit (Affymetrix), as per the manufacturer's instructions. Double-stranded cDNA products were purified using the GeneChip® Sample Cleanup Module (Affymetrix). The synthesised cDNAs were transcribed in vitro by T7 RNA polymerase using biotinylated nucleotides to generate biotinylated complementary RNAs (cRNAs) using the GeneChip® HT IVT labelling kit (Affymetrix), according to the manufacturer's instructions. The cRNAs were purified using the GeneChip® Sample Cleanup Module (Affymetrix). The cRNAs were then randomly fragmented at 94 °C for 35 min in a buffer containing 40 mM Tris–acetate (pH 8.1), 100 mM potassium acetate and 30 mM magnesium acetate to generate molecules of approximately 35–200 bp. Affymetrix *C. elegans* Genome GeneChip® arrays were hybridised with 15 µg of fragmented labelled cRNA for 16 h at 45 °C as described in the Affymetrix Technical Analysis Manual using the GeneChip® hybridisation control kit and GeneChip® hybridisation, wash and stain kit (Affymetrix). GeneChip® arrays were stained with streptavidin–phycoerythrin solution and scanned with an Affymetrix G2500A GeneArray scanner. Following

scanning, non-scaled RNA signal intensity (CEL) files were generated using GeneChip® operating software (GCOS; Affymetrix) and normalised data were generated with the GCOS software using the MAS 5 algorithm (Affymetrix Microarray Suite User Guide: http://www.affymetrix.com/support/technical/manuals.affx). The ratios of 5′ to 3′ sequence representation on the final gene arrays were checked for several housekeeping transcripts (catalase, GAPDH, Gly4, ubiquitin and actin): for GAPDH and actin these were mostly close to unity, for catalase they were around 0.5, while for Gly4 they were much higher (4.0 to nearly 5.0) and for ubiquitin much lower (around 0.2). These ratios were fairly constant across all of the different RNA samples, suggesting that these differences in 5′:3′ ratios reflect differential probe efficiency. The most divergent ratios were those seen for sham 1.

GFP Reporter Expression Analysis

Three transgenic strains carrying integrated GFP fusion genes were chosen from a panel of stress-inducible strains. One of these (CL2070) carries an *hsp*-16.2::GFP fusion gene known to be inducible by mild heat (at 30 °C), whereas previous work [Anbalagan and de Pomerai, unpublished work] suggested that neither *daf*-16::GFP (TJ356) nor *cyp*-34A9::GFP (BC20306) fusion genes is heat-inducible (all three are inducible by other stressors). Cultures of each strain were grown at 15 °C, washed with ice-cold K medium as above and aliquotted equally in liquid K medium (with constant gentle stirring to prevent worms from settling) into 24-well plates using 0.3 ml per well (containing about 500 worms). These liquid cultures were exposed for up to 6 h at either 26 °C (control) or 30 °C (heat shock). Temperature records for this extended run and for three shorter 1.5 h runs (used for RNA preparations) are shown in Supplementary Material 2; in essence, these set temperatures correspond to measured actual values of 25.5 °C (occasionally rising to 26.0 °C) and 30.0 °C (overshooting to 30.5 °C initially, then settling back to 30.0 °C and occasionally dropping to 29.5 °C). Thus a temperature disparity of 4–5 °C was maintained throughout between the control and heat-shock conditions. After 3 h, and again after 6 h, the contents of each well were transferred to a 96-well, black, non-fluorescent microplate with round-bottomed wells (Nunclon, Cole-Parmer Instruments, Hanwell, London, UK) and the worms were allowed to settle on ice for 10 min. GFP fluorescence was measured in each worm pellet using a Perkin-Elmer Victor 1420 plate fluorometer with excitation and emission filters for GFP. Because the *daf*-16::GFP strain showed much higher constitutive GFP expression than the other two strains, all GFP measurements (in relative fluorescence units, RFU) have been normalised to the 3 h control value (at 26 °C) for each strain. Each bar in Figure 3 shows the mean and standard error derived from four independent replicates for that strain.

Data Analysis

The non-scaled RNA CEL files were loaded into GeneSpring analysis software (GeneSpring 7.3; Agilent Technologies) using the Robust Multichip Average (RMA) prenormalisation algorithm [Irizarry et al., 2003]. Further normalisations were performed for each experiment using a three-step process: (i) probe sets with a signal value <0.01 were set to 0.01, (ii) per chip normalisation to the 50th percentile, (iii) each gene signal from the microwave-treated sample was normalised to the corresponding sham sample and the sham samples normalised to themselves. Raw *P*-values obtained from paired *t*-tests were evaluated in the light of the high probability of making a 'false discovery' [Storey and Tibishrani, 2003]. We have therefore adjusted the *P*-values using a standard correction for instances of multiple testing [Benjamini and Hochberg, 1995]. The minimum false discovery rate at which each *P*-value could be described as significant (the *q*-value) was estimated using the *q*-value v1.0 library implemented in the statistical package R, v 2.4.1. The data discussed in this publication are accessible in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) [Edgar et al., 2002] through the GEO series accession number GSE10787.

RESULTS

A schematic plan of the Nottingham TEM cell is shown in Figure 1A, indicating the central position of the 24-well plate used for sample exposures in previous studies [Dawe et al., 2006]. Figure 1B shows the measured SAR distribution with a loaded 24-well plate (each well containing 1.0 ml of K medium) for a 0.9 GHz CW signal at 1.0 W input power, using the NPL measurement equipment and conditions described in the Materials and Methods Section. SAR was highest in the input corner wells (up to $\sim$40 mW kg^{-1}) and lowest in the central wells ($\sim$4 mW kg^{-1}). However, because very large numbers of sham and exposed worms were required for RNA preparations and gene-array analysis in this study, we minimised potential problems from anoxia and overcrowding by conducting the exposures in 6-well plates. Similar SAR measurements were not conducted in this 6-well plate format, but SAR modelling by FDTD was performed for both 6- and 24-well formats. Figure 2A confirms that peak SAR in the middle layer of K medium (halfway between

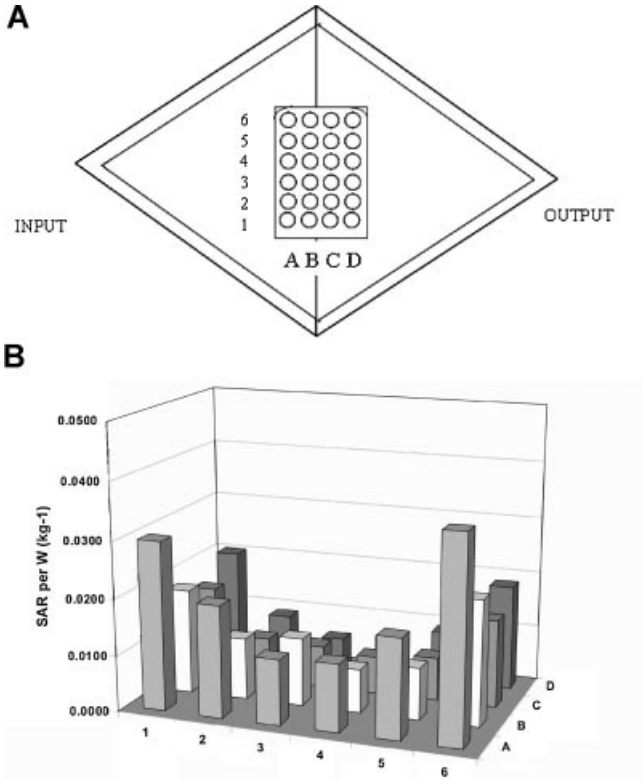

Fig. 1. Measured SAR distribution in K medium for a 24-well plate. **Part A** shows a schematic cross-section of the octahedral Nottingham TEM cell, with a 24-well multiwell plate placed centrally on the waveguide septum. The SAR measurements shown in **part B** were performed as described in Materials and Methods Section, using an isotropic IndexSAR IXP-010 E-field probe dipping into 1.0 ml of K medium (midway between meniscus and floor), which had been dispensed beforehand into each well of the 24-well plate. In this experiment, a 0.9 GHz CW field was applied, and results were normalised to a 1.0 W power input (NB these conditions differ somewhat from those used in the simulations shown in Figure 2, and in the gene-array experiments described here).

meniscus and floor, as in Fig. 1B), is essentially confined to the corner wells, with an overall variation from about 2 to 20 mW kg^{-1} (for a 0.5 W input, rather than 1.0 W as in Fig. 1B). Thus SAR measurements for the 24 -well format are generally in close agreement with FDTD modelling and also with independent TLM modelling [Vasic and Thomas, unpublished work; data not shown]. FDTD modelling predicts a somewhat higher and more uniform SAR in the basal layer of K medium for the 24-well format, as shown in Figure 2B. However, we note that FDTD modelling predicts ninefold lower SAR for this same basal layer in the 6-well compared to the 24-well format (Fig. 2C vs. Fig. 2B). Normalised to 0.5 W of average power supplied to the TEM cell at 1.0 GHz, simulations for the basal layer of K medium in 24-well plates gave a peak 1 g average SAR of 8.2 mW kg^{-1}, a total average SAR in K medium of 4.7 mW kg^{-1} and the peak point in the exposed medium was 25 mW kg^{-1} (Fig. 2B). For the 6-well exposure system as used here, similar modelling gave a peak 1 g average SAR of 1.0 mW kg^{-1}, a total average SAR of 0.88 mW kg^{-1} and the peak point in exposed K medium was 2.7 mW kg^{-1} (Fig. 2C). These SAR estimates relate to average voxel values centred 0.25 mm above the floor of each well, since this basal layer of K medium is where worms ($\sim$100 µm in diameter) spend most of their time resting or crawling. SAR modelling for other layers of medium is available as Supplementary Material 1, but detailed modelling of the meniscus region requires higher resolution and is in any case not relevant to worms residing mainly in the basal layer.

The actual SAR experienced by worms crawling across the well floor in a microwave-exposed 6-well multiwell plate is therefore likely to vary from $<$1.0 mW kg^{-1} in central wells up to a maximum of 2.7 mW kg^{-1} in the corner wells (at 0.5 W input power). Clearly the RNA extraction and gene array procedures pool worms from all exposed wells together and compare these against unexposed sham controls. Even when using the 24-well format (where SAR is $\sim$9-fold higher; up to 25 mW kg^{-1} in corner wells), we have been unable to detect any significant differences in *hsp*-16.1::*lacZ* expression between corner and central wells after microwave exposure [Dawe and de Pomerai, unpublished work].

Table 1 lists the genes that showed possible up- or down-regulation (by $\geq$20%) following microwave exposure, ordered by *n*-fold change in expression relative to sham controls after standard normalisation (see Materials and Methods Section). The maximum increase observed is only 40% (for F40F12.5, encoding an orthologue of human CYLD1). One gene that was variably up-regulated in three out of five runs (albeit by only 17% on average) is the F59D8.1 locus including two linked vitellogenin (yolk-protein) genes, *vit-3* and *vit-4*. At first sight, this might be consistent with a previous report that microwave exposure can speed up egg production in *C. elegans* [de Pomerai et al., 2002], although this observation could also be plausibly explained by slight heating. Vitellogenin (*vit*) genes are expressed in the adult gut to facilitate egg production, but not in L4 larvae (which produce only sperm in the hermaphrodite gonad); thus the onset of *vit* gene expression is diagnostic of the transition from L4 to adulthood. However, the *vit-3/-4* locus is part of a vitellogenin multigene family comprising five closely related X-linked genes (*vit-1* to *-5*) plus one distantly related autosomal gene (*vit-6*) [Heine and Blumenthal, 1986]. Most are co-regulated in the adult intestine,

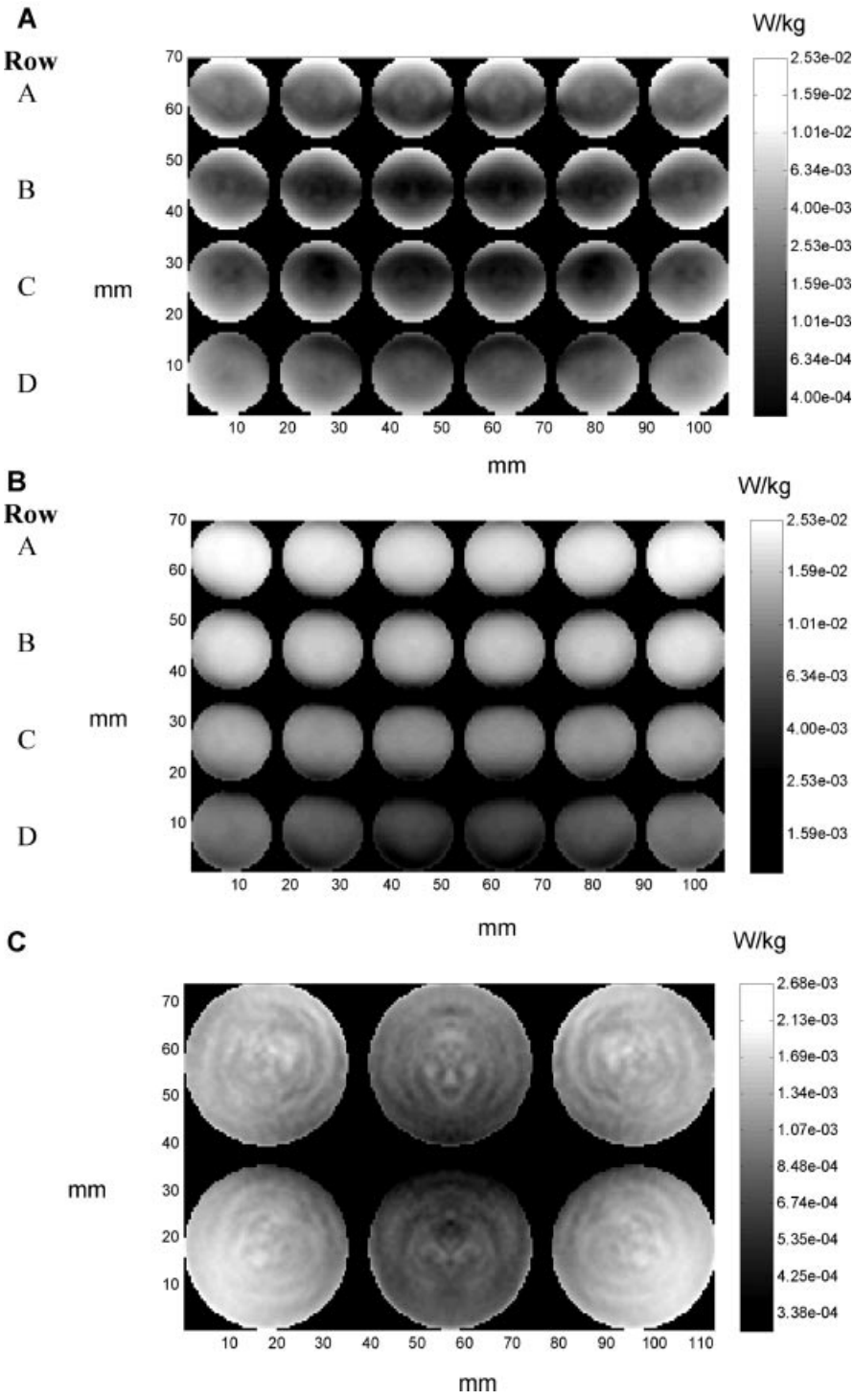

Fig. 2. Modelling of SAR distributions for 24- and 6-well plates in K medium. Numerical computer simulations were used to simulate SAR for 1.0 GHz CW fields at 0.5 W input power in 1.0 ml samples of K medium dispensed into each well of a 24-well (**parts A,B**) or 6-well (**part C**) plate inside the TEM cell as above (Fig. 1A). The computations were performed using the XFDTD commercial software tool (Remcom), as described in the Material and Methods section, and the geometry of the simulation model was as close as possible to the actual experimental setup. The SAR estimates are averaged for each 0.5 mm^3 voxel in either: (i) the middle layer of K medium (midway between meniscus and floor, corresponding to the probe location in Fig. 1B) as shown in part A; or (ii) the basal layer of K medium above the well floor in both plate formats (centred 0.25 mm above the floor) as shown in parts B and C. The greyscale bar on the right of each figure shows the SAR in W kg^{-1}. Each plate is shown in the same orientation with row A (closest to input; Fig. 1A) uppermost.

TABLE 1. Genes Showing Significant ($P \leq 0.05$) Expression Changes of ≥20% in Microwave-Exposed Compared to Sham Controls, Based on Pairwise Comparisons of all Five Replica Gene Arrays for Each Condition

Gene name and/or identifier	*n*-fold change	*P*-value	Inferred biological or molecular function	Gene ontogeny term(s)
F40F12.5 (3K988)	1.405	0.048	Human CYLD1 orthologue (NFκB signalling)	Cellular signalling?
F54D11.2 (5F105)	1.31	0.037	α/β hydrolase	Embryonic development
Y67D8A.1 (4E15)	1.30	0.007	Phosphorylase kinase β subunit	Glycogen metabolism
Y69A2AR.16 (4D293)	1.28	0.006	Exportin 4—nuclear export	Metabolic process
T14G11.3	1.27	0.030	Mitochondrial inner membrane protein	Membrane organisation
Y41D4B.19 *npp-8*	1.27	0.040	Nuclear pore complex protein	Nucleocytosplasmic transport; embryonic cleavage
Y57G11C.15 *sec-61α*	1.25	0.046	Transport protein	Protein secretion
T22B11.5 (4F462)	1.24	0.011	Mitochondrial 2oxoglutarate dehydrogenase	Glycolysis
Y39H10A.3 *mtm-9*	1.24	0.044	Myotubularin—endocytosis in coelomocytes	Endocytosis
F52B11.3 *noah-2*	1.24	0.039	Essential for mechanoreceptor potential	Embryonic development
Y45G5AM.9 (5E660)	1.23	0.043	Attractin and platelet-activating factor acetylhydrolase	Putative nuclear protein?
Y59A8A.3	1.23	0.042	Protein with four coiled coil domains—putative involvement in transport to Golgi apparatus	Protein trafficking?
ZK1073.1 (XQ738)	1.22	0.049	NDR-1—essential for cell differentiation	Cell differentiation
C27C7.1	1.22	0.006	Putative nuclear protein	Putative nuclear protein?
T05D4.1 (3O652)	1.22	0.038	Fructose-1,6-bisphosphate aldolase	Glycolysis
B0334.5	1.22	0.025	Phytoene/squalene synthetase	Regulation of growth rate
C16C10.11	1.22	0.038	Mitochondrial carrier protein; EtOH-induced	Energy metabolism?
M01D7.7 *egl-30*	1.21	0.010	Heterotrimeric G protein α subunit	Signal transduction
F38A6.3 *hif-1*	1.21	0.038	Hypoxia-inducible transcription factor	Transcriptional regulation
W02D9.3	1.20	0.008	HMG-box protein	Chromosome segregation
F59D8.1/.2 *vit-3/-4*	1.17	0.002	Major adult yolk proteins (vitellogenins)	Embryo dev.; lipid transport
F36F2.2 (IJ593)	0.85	0.029	Uncharacterised protein	Not assigned
ZC455.10 *fkb-4*	0.85	0.023	FKBP-type peptidyl-prolyl cis-trans isomerase	Protein modification
C54F6.14 *ftn-1*	0.84	0.015	Ferritin 1 heavy chain	Iron homeostasis
B0454.10 *sri-30*	0.84	0.05	Class I serpentine G-protein coupled receptor	Chemosensory?
F45C12.3 (2C19)	0.84	0.04	Homeodomain transcription factor	Transcriptional regulation
W02B3.3 (3B284)	0.82	0.031	Uncharacterised protein	Not assigned

Several transcripts showing similarly altered expression do not correspond to any known (or annotated) *C. elegans* gene, and these have therefore been omitted.

being subject to repression by the *double-sex*-related MAB-3 transcription factor, which is in turn negatively regulated by the TRA-1 sex-determining transcription factor [Shen and Hodgkin, 1988; Yi et al., 2000]. However, none of the other *vit* genes showed any significant change in expression between sham and exposed conditions (expression ratios of 0.9–1.1) in any of the five runs. Thus the apparent up-regulation of *vit-3/-4* is unlikely to be of any significance biologically.

As a positive control, we heat-shocked N2 worms for 1.5 h at 30 °C and compared duplicate gene arrays against parallel 26 °C sham controls (for each run, two batches of N2 *C. elegans* L4 larvae were split equally between the 26 and 30 °C conditions prior to exposure). Comparisons between the two heat-shock gene arrays at 30 °C and their respective controls at 26 °C reveal a large number of gene expression changes that appear significant both statistically ($P \leq 0.05$) and quantitatively (≥2-fold change). Prominent among the up-regulated genes are those encoding several heat-shock proteins, notably a major inducible *hsp*-70 (C12C8.1; 16.6-fold) and *hsp*-16.2 (7.2-fold). Unfortunately, these changes do not stand up to further statistical scrutiny after applying the Benjamini–Hochberg correction and could therefore represent false positives (see Discussion Section). This is largely a consequence of discrepancies between the two 26 °C sham arrays (see Supplementary Material 3); by contrast, the two 30 °C heat-shock arrays show very similar patterns of altered gene expression. We therefore conducted a further comparison between the two 30 °C heat-shock gene arrays and the original set of five 26 °C sham arrays from the main microwave study (noting that these were exposed for 2.5 h rather than 1.5 h at 26 °C). This indicates that 1585 genes show significantly altered expression at 30 °C compared to 26 °C, with $P \leq 0.05$ even after applying the Benjamini–Hochberg correction; 556 of these are down-regulated by ≥2-fold, while 1029 are up-regulated by ≥2-fold. Table 2 lists those genes showing ≥10-fold up-regulation and ≥6.67-fold down-regulation. The latter group mostly encode uncharacterised proteins, of which 25% (3 out

TABLE 2. Genes Showing Significant Expression Changes ($P \leq 0.05$ After FDR) in Heat-Shocked (30 °C for 1.5 h; Two Arrays), Compared to Sham Controls (26 °C for 2.5 h; Five Arrays)

Gene name and/or identifier	*n*-fold change	*P*-value	Inferred biological/molecular function	Gene ontogeny term(s)
C12C8.1 major *hsp*-70	94.9	0.009	Major inducible heat-shock protein	Protein trafficking and repair
F44E5.4 major *hsp*-70	63.0	0.004	Major inducible heat-shock protein	Protein trafficking and repair
ZK666.6 *clec*-60	59.1	0.0003	C-type lectin	Cell-surface carbohydrate binding
C45G7.3 *ilys*-3	39.1	3×10^{-5}	Invertebrate lysozyme (inducible by *Microbacterium nematophilum* infection)	Antibacterial lysozyme
F22B5.3 *cut*-3	35.1	3×10^{-5}	Cuticulin family member	Cuticle formation
T05H10.3	30.9	0.001	Permease of major facilitator superfamily	Transport across cell membranes
Y53G8AM.5	27.6	0.008	Putative secreted/extracellular protein?	Not assigned
H27M09.4 *dpy*-14/*col*-59	26.1	0.004	Type IIIα1 collagen	Cuticle formation
C08B6.4b	25.0	0.036	Chtinase	Catabolism
F19B2.5	22.4	0.009	Similarity to mouse helicase-like TF	Not assigned
T22G5.7 *spp*-12 (*dod*-5)	21.5	0.01	Saposin-like protein	Sphingolipid metabolism (?)
F54D8.1 *dpy*-17	20.3	0.003	Collagen α precursor	Cuticle formation
F17E9.11 *lys*-10	19.5	0.002	Lysozyme family member	Antibacterial lysozyme
E03H4.10 *clec*-17	16.5	0.005	C-type lectin	Cell-surface carbohydrate binding
Y46H3A.3 *hsp*-16.2	16.3	0.014	Small heat-shock protein family member	Prevention of protein aggregation
F36D3.9 *cpr*-2	15.3	0.035	Cysteine-type endopeptidase	Protein catabolism
ZK1248.2 *col*-74	14.8	0.009	Collagen	Cuticle formation
F40E10.1 *hch*-1/*aas*-34	13.6	0.045	Metallopeptidase? (required for hatching)	Protein catabolism
C02B4.2 *nhr*-17	13.1	0.0003	Nuclear hormone receptor family member	Transcriptional regulation
C54D1.2 *clec*-86	12.9	0.008	C-type lectin	Cell-surface carbohydrate binding
F53B3.5	12.6	0.003	Claudin (with four transmembrane domains)	Integral membrane protein
Y110A2AL.4	11.9	0.0003	Uncharacterised protein	Not assigned
F56D5.1 *col*-121	11.7	0.003	Collagen	Cuticle formation
C45G7.2 *mboa*-3	11.6	0.032	Membrane-bound *O*-acyltransferase	Membrane protein
T27E4.2 *hsp*-16.11	11.3	0.011	Small heat-shock protein family member	Prevention of protein aggregation
W03G1.7 *asm*-7	10.8	0.012	Acid sphingomyelinase	Sphingolipid metabolism
Y41C4A.11	10.5	0.0017	Paralog of β′ subunit of COPI complex	Vesicle trafficking
F55C10.3 *col*-165	10.4	0.012	Collagen	Cuticle formation
F37B1.4 *gst*-15	10.0	0.0003	Glutathione-*S*-transferase	Phase II xenobiotic metabolism
ZK484.8 *nspd*-1	0.15	0.010	Uncharacterised nematode-specific protein	Not assigned
C33F10.1	0.15	0.007	Uncharacterised protein (membrane?)	Not assigned
C24D10.7 *nspd*-3	0.15	0.008	Uncharacterised nematode-specific protein	Not assigned
Y67D8C.8	0.14	0.025	Chondroitin proteoglycan	Extracellular matrix
T28D6.3	0.14	0.002	Uncharacterised protein	Not assigned
F32A5.2a	0.14	0.003	Peroxidase/oxygenase	Oxidative stress response
F41H10.7 *elo*-5	0.14	0.003	PUFA elongase	Fatty acid metabolism
C27D6.3	0.14	0.013	Uncharacterised protein	Not assigned
T23B7.1 *nspd*-4	0.14	0.008	Uncharacterised nematode-specific protein	Not assigned
C04F12.7	0.12	0.025	Uncharacterised protein	Not assigned
ZK512.7	0.10	0.014	Uncharacterised protein	Not assigned
T13F3.6	0.09	0.009	Uncharacterised protein	Not assigned

Only those genes that are ≥10-fold up-regulated or ≤6.67-fold down-regulated are listed here. See text for details.

of 12) are nematode-specific (*nspd*-4, -3 and -1). The larger group of strongly up-regulated genes includes two *hsp*-70 genes (C12C8.1 by 95-fold; F44E5.4 by 63-fold) and two small heat-shock genes (*hsp*-16.2 by 16.3-fold; *hsp*-16.11 by 11.3-fold). Other heat-shock genes showing significant up-regulation (between 2- and 10-fold) include: *hsp*-16.41 by 8.2-fold, *hsp*-17 by 8.0-fold, *hsp*-43 by 3.9-fold, *hsp*-16.48 by 2.8-fold, *hsp*-3 by 2.4-fold and *hsp*-4 by 2.1-fold. Other genes that figure prominently in the up-regulated group include several collagen genes (*dpy*-14, *dpy*-17, *col*-74, *col*-121 and *col*-165) and a cuticulin gene (*cut*-3), presumably reflecting heat-induced changes in cuticle synthesis (Table 2). Three C-type lectin genes (*clec*-60, *clec*-17 and *clec*-86) and two lysozyme genes (*ilys*-3 and *lys*-10) are also up-regulated strongly at 30 compared to 26 °C (Table 2).

We have independently confirmed some of the positive control gene-array results by monitoring GFP expression in three transgenic strains carrying stress-related promoter::GFP fusion genes. As expected, the *hsp*-16.2::GFP construct is strongly up-regulated by

mild heat shock at 30 °C—by ~25% after 3 h and by ~200% after 6 h (Fig. 3). This response appears slower and lower than implied by the gene-array results (7.2-fold), but in fact a considerable proportion of the background 'GFP' signal at 26 °C is contributed by gut autofluorescence rather than low-level fusion-gene expression (hence the true extent of up-regulation is partially hidden by this background signal noise). Moreover, the GFP response is delayed relative to transcriptional changes in gene expression, because the GFP protein needs to be translated, correctly folded and auto-oxidised before any increase in fluorescence can be detected (a matter of hours rather than minutes). Thus, in essence, the results for heat-inducible *hsp*-16.2::GFP expression (Fig. 3) confirm the gene-array data shown in Table 2. Similar experiments on strain PC161 (an *hsp*-16.1::GFP:*lacZ* double reporter strain) [David et al., 2003] revealed a similar but smaller up-regulation after 6 h at 30 °C (data not shown). Figure 3 also confirms that two other stress-related genes, *daf*-16 (encoding a FOXO transcription factor central to the ageing pathway) and *cyp*-34A9 (=*dod*-16, a cytochrome P450 gene which is itself regulated by DAF-16) [Murphy et al., 2003; de Pomerai et al., 2008], show only marginal down-regulation at 30 °C (approaching twofold for *daf*-16 after 6 h). The gene array data for these two genes suggest slight up-regulation for *cyp*-34A9 (2.18-fold) and no change for *daf*-16 (although there are multiple transcripts from this gene and three of the four probe sets are not specific for *daf*-16). Broadly speaking, these independent controls confirm strong up-regulation for *hsp*-16.2 but only minor changes for *daf*-16 and *cyp*-34A9.

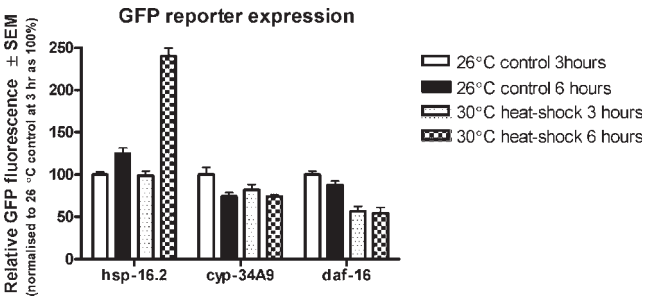

Fig. 3. Effect of heat shock at 30 °C on expression of selected GFP reporters. Equal aliquots of transgenic worm strains carrying integrated *hsp*-16.2::GFP (CL2070), *daf*-16::GFP (TJ356) and *cyp*-34A9::GFP (BC20306) constructs were exposed at 26 or 30 °C for up to 6 h, and the GFP fluorescence measured after 3 and 6 h, as described in Materials and Methods Section. Relative fluorescence was normalised relative to the basal expression at 26 °C after 3 h for each test strain. Each histogram bar shows the mean and SEM derived from four independent replicates. Each group of four bars (one strain) shows, from left to right, the relative GFP fluorescence at: −26 °C after 3 h (set at 100%; no shading); 30 °C after 3 h (solid shading); 26 °C after 6 h (speckled); and 30 °C after 6 h (chequered).

DISCUSSION

The apparent changes in gene expression between matched sham and microwave-exposed samples are in all cases quantitatively small (Table 1). Such changes are rarely consistent across all five runs and mostly occur in only three (sometimes four) out of the five. Moreover, the known or inferred functions and gene ontogeny terms for the listed genes do not suggest any particular common targets. None of these apparent gene-expression changes is sufficiently large or consistent to justify real-time RT-PCR measurements of transcript levels in order to confirm a genuine change in gene-expression levels. A modest 4–5 °C increase in temperature provokes far more numerous changes in gene-expression profile, many of which are quantitatively much larger. This shows that the Affymetrix gene arrays used here can clearly identify major gene-expression changes provoked by fairly mild environmental perturbations. Furthermore, using GFP fusion strains, we have validated at least one case of significant up-regulation at 30 °C (*hsp*-16.2) and confirmed that two other stress-related genes (*daf*-16 and *cyp*-34A9) are scarcely affected by mild heat (Fig. 3).

Given the fact that Affymetrix *C. elegans* gene arrays measure the levels of >22000 transcripts and that 5% of these are likely to show apparently significant ($P < 0.05$) changes in expression levels by chance, we would expect to see ~1131 false discoveries under the null hypothesis that microwave exposure has no effect on gene expression levels in *C. elegans*. In reality, we observed even fewer apparently significant changes after microwave irradiation, as only 846 of the tests yielded $P < 0.05$. The q-values that we computed indicate that, if we were to reject the null hypothesis for any one of these 846 tests, the chance of making a false discovery would be at least 64% ($q > 0.641$ for all entries in Table 1). Furthermore, if we adjust the P-values using a standard correction for instances of multiple testing [Benjamini and Hochberg, 1995], none of the observed gene-expression changes remains significant ($P = 0.873$ for all entries in Table 1). Thus, on statistical grounds, there is no reason to reject the null hypothesis for any of the genes considered. Whilst it is impossible to prove a negative (i.e. that there is no effect whatsoever), this study provides no clear evidence for microwave-induced changes in gene expression. Our negative conclusion is reinforced by the fact that five pairwise comparisons between exposed and sham conditions (each pooled from 3 separate runs, i.e., representing 15 runs in total) have failed to pinpoint any

consistent or significant changes in gene expression beyond those predicted by chance. This conclusion is unaffected by the inclusion or exclusion of two data sets (from runs one and three) that showed somewhat higher variance than in runs two, four or five. In fact, exposed and sham arrays from each run were generally more similar to each other than to the remaining four exposed or sham arrays (see condition tree plots presented as Supplementary Material 3A).

We also undertook a parallel study using mild heat -shock conditions (30 °C) for comparison against 26 °C controls (Table 2). Because heat induces rapid but transient changes in gene expression (especially for heat-shock genes) [GuhaThakurta et al., 2002], we chose a shorter 1.5 h exposure time rather than the 2.5 h used for microwave exposures (above). Though 30 °C is well above the normal tolerance range for *C. elegans*, it is milder than the standard heat-shock conditions (33 or 35 °C) tested in other gene-array experiments [Kim et al., 2001; GuhaThakurta et al., 2002]. Comparisons between the two 30 °C arrays and the corresponding 26 °C arrays reveal numerous up- and down-regulated genes, but unfortunately these do not remain significant after applying the Benjamini–Hochberg correction, suggesting that some or most may be false positives. The main reason for this is an unexplained disparity between the two 26 °C sham arrays, in contrast to the two 30 °C arrays which are closely similar (see condition tree plots presented in Supplementary Material 3B). We therefore re-analysed the gene-array data by comparing the two 30 °C arrays against the original set of five 26 °C sham controls from the main microwave experiment. This analysis reveals a total of 1585 changes in gene expression that are $\geq$2-fold and remain significant after applying the Benjamini–Hochberg correction. Amongst the most strongly up-regulated genes are several encoding heat-shock proteins, cuticle components, C-type lectins and lysozymes (see Table 2). Thus a few functional categories predominate amongst the heat-inducible genes, in contrast to the very mixed bag showing marginal changes in response to microwave irradiation (Table 1).

In conclusion, this study provides no evidence for major changes in gene-expression following exposure of wild-type *C. elegans* to weak microwave fields (2.5 h at 26 °C; SAR 0.9–2.7 mW kg^{-1}). The slight changes observed are quantitatively small and likely to represent false positives. The fact that far more genes show marginal up-regulation than down-regulation might be consistent with the very small residual temperature rise ($\sim$0.1 °C) experienced within the modified TEM cell during microwave exposure [Dawe et al., 2006], since a similar preponderance of up-regulated genes over down-regulated genes is also seen at 30 °C (Table 2). This does not exclude the possibility that some genes may show altered expression following exposure to stronger fields (of the order of 2.0 W kg^{-1}, as used in many other studies), nor that susceptible mutant strains might show greater sensitivity. However, despite its many advantages of convenience and excellent genetics, *C. elegans* may be too resilient as a test organism to offer sensitive biomarkers for microwave exposure and effect.

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