Effects of radio-frequency fields on bacterial cell membranes and nematode temperature-sensitive mutants.

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ABSTRACT.

Membrane-related bioeffects have been reported in response to both radio-frequency (RF) and extremely low-frequency (ELF) electromagnetic fields (EMFs), particularly in neural cells. We have tested whether RF fields might cause inner membrane leakage in ML35 *E. coli* cells, which express β-galactosidase (*lacZ*) constitutively, but lack the *lacY* permease required for substrate entry. The activity of *lacZ* (indicating substrate leakage through the inner cell membrane) was increased only slightly by RF exposure (1 GHz, 0.5 W) over 45 min. Since *lacZ* activity showed no further increase with a longer exposure time of 90 min, this suggests that membrane permeability *per se* is not significantly affected by RF fields, and that slight heating (≤ 0.1°C) could account for this small difference. Temperature-sensitive (ts) mutants of the nematode, *Caenorhabditis elegans*, are wild-type at 15°C but develop the mutant phenotype at 25°C; an intermediate temperature of 21°C results in a reproducible mixture of both phenotypes. For two ts mutants affecting transmembrane receptors (TRA-2 and GLP-1), RF exposure for 24 h during the thermocritical phase strongly shifts the phenotype mix at 21°C towards the mutant end of the spectrum. For ts mutants affecting nuclear proteins, such phenotype shifts appear smaller (PHA-1) or non-significant (LIN-39), apparently confirming suggestions that RF power is dissipated mainly in the plasma membrane of cells. However, these phenotype shifts are no longer seen when microwave treatment is applied at 21°C in a modified exposure apparatus that minimises the temperature difference between sham and exposed conditions. Like other biological effects attributed to microwaves in the *C. elegans* system, phenotype shifts in ts mutants appear to be an artefact caused by very slight heating.

INTRODUCTION.

Controversy and contradiction still surround the question of whether electromagnetic fields (EMFs) cause any detectable biological effects, other than those attributable to heating at higher frequencies (microwave or RF range at 10^8-10^{11} Hz, e.g. radiation from mobile phones), or to induced electric currents at extremely low frequencies (ELF range at 1-1000 Hz, e.g. surrounding power lines). Several reports have adduced evidence for such effects – both harmful and beneficial – but the vast majority have found no effect whatsoever. Even *bona fide* attempts at replication of positive results have often foundered on the rocks of irreproducibility. A case in point is the key study by Repacholi *et al* (1) suggesting that long-term RF exposure can increase lymphoma rates in Eμ-*Pim1* transgenic mice, an effect which could not be reproduced in a recent replication study by Utteridge *et al* (2). The fact that this second study was not an exact repeat, but made several changes to the complex protocol, makes these opposing findings even more difficult to reconcile. There is an urgent need to develop simple and cheap assays for EMF bioeffects, which should both improve statistical reliability (since numerous replicates would be practicable) and also reduce the likelihood of variant procedures leading to different outcomes. Such assays have the potential to be robust and repeatable, although any such conclusion must await replication in other laboratories.

We have studied the effects of RF (750 MHz or 1 GHz and 0.5 W; SAR range 15-40 mW kg^{-1}) on stress-inducible transgenic strains of the model nematode, *Caenorhabditis elegans*, and found evidence suggesting non-thermal RF induction of heat-shock reporters at 25°C (3, 4), as well as effects on growth and egg production (5). Two groups have independently reported similar heat-shock responses to ELF magnetic fields in the same or related *C. elegans* strains (6, 7), confirming their utility for such studies. Heat-shock responses can be induced by a wide range of stressors (8), most of which act by causing conformational damage to cellular proteins. Damaged polypeptides bind to pre-existing heat-shock proteins, which dissociate from their cytoplasmic complexes with the heat-shock transcription factor, HSF. Released HSF monomers rapidly undergo trimerisation, phosphorylation and translocation into the nucleus, so triggering transcription of inducible heat-shock genes (9). Thus, exposure to RF or ELF EMFs may possibly lead to protein conformational damage, either directly or indirectly through a co-stressor which is not in itself sufficient to trigger a heat-shock response. The strong temperature dependence of RF-induced responses (4, 6) suggests that heat itself may be one such co-stressor. Although small temperature differences (0.1-0.3°C) are often dismissed as irrelevant in EMF studies, recent work in our laboratory suggests otherwise (10).

We have shown that our RF exposure system introduces a small temperature disparity, such that RF-exposed samples are ≤ 0.2°C warmer than sham-exposed samples, as a result of power losses within the live cell. This adventitious sample heating can be reduced (but not eliminated) by modifying the exposure cell; however, there is no detectable RF induction of heat-shock reporter expression in the modified cell (10). Confirmation that the induction originally seen in the unmodified cell was indeed a thermal artefact has come from heating studies, showing that a temperature rise of only 0.2°C is sufficient to induce comparable expression of the same heat-shock reporter construct (10).

Several different RF bioeffects involving the cell plasma membrane have been described in the literature, including altered neuronal firing patterns and changes in ion fluxes (especially Ca^{++}), as well as erythrocyte leakage (reviewed 11). Very few of these effects have been replicated in other laboratories. We have used a somewhat different approach involving the ML35 *E. coli* strain, which constitutively expresses the β-galactosidase (*lacZ*) enzyme but does not possess the relevant permease enzyme (*lacY*) to transport β-D-galactopyranoside substrates across the inner bacterial membrane. When ML35 cells are incubated in substrate solutions, β-galactosidase activity is restricted by the modest rate of substrate leakage into the cells. However, any damage to the inner bacterial membrane – caused e.g. by detergents or pore-forming toxins – results in significant increases in enzyme activity because more substrate enters the cells (12). We have therefore used this simple bacterial assay to investigate whether RF (or ELF electrical) fields might affect membrane fluidity, bearing in mind that this parameter is also strongly temperature-dependent. The very modest increases in enzyme activity observed here are unaffected by a longer exposure time and probably point to a very slight temperature increase (≤ 0.2°C) as the underlying cause. This is consistent both with our own temperature measurements (3, 4) and with traceable calibration of our exposure system by the UK National Physical Laboratory (10).

We also examine the effects of RF exposure on intrinsically thermolabile proteins encoded by temperature-sensitive (ts) mutant genes in *C. elegans*. Such mutants develop into wild-type worms at 15°C but show the mutant phenotype at 25°C. Intermediate temperatures (20-22°C) result in a mixture of both phenotypes in reproducible proportions. We chose mutants with easily scored, distinctive phenotypes (see Methods). The fact that different ts protein products are located in different subcellular compartments provides an added bonus. In particular, we wished to test the recent model of Kotnik and Miklavcik (13), suggesting that microwave power should be dissipated mainly in the cell plasma membrane. If true, ts mutants affecting transmembrane proteins should be

strongly shifted towards the mutant end of the spectrum following RF exposure during the thermocritical period at an intermediate temperature. As a corollary, much smaller shifts should be apparent for ts mutants affecting nuclear or cytoplasmic proteins. This prediction apparently holds true for ts mutants affecting two nuclear transcription factors as compared to two transmembrane receptors. However, further investigations indicate that these phenotype shifts are lost when the RF exposures are conducted in the modified cell where heating is minimised . We conclude that some ts mutant phenotypes are exquisitely sensitive to small changes in temperature, but that there is no independent effect of weak RF fields *per se* on protein conformation, even in the plasma membrane.

MATERIALS AND METHODS.

The ML35 *E. coli* strain was donated by Prof. R. Hancock (Dept. of Microbiology & Immunology, University of British Columbia, Vancouver, Canada), and ts strains of *C. elegans* were supplied by the *Caenorhabditis* Genetics Centre (Minneapolis, USA). Non-fluorescent black plastic 96-well microplates were from Corning-Costar (Corning Inc, New York, USA), and all chemical reagents were from Sigma (Poole, Dorset, UK). Heating/cooling incubators were model LT3 from LEEC Ltd (Nottingham, UK); their accuracy (± 0.2°C) was checked with narrow-range Hg thermometers. For the later studies shown in Table 1, both the sham and modified RF-exposure cells (see below) were housed on the same shelf of a water-jacketed Sanyo MIR-553 incubator set at 21.0 ± 0.1°C.

Worm culture methods and phenotype assessments: Temperature-sensitive worm strains (DH202, MT1514, GE1076 and JK509) were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis). All strains were grown at 15°C on standard NGM agar plates (14) plated with *E. coli* OP50 as food bacteria (grown up overnight at 37°C). In some cases (particularly for DH202 and MT1514), we used higher concentrations of peptone (10 g l^{-1}) in the NGM agar to achieve denser bacterial lawns, with the aim of promoting optimal worm growth. Worms transferred from stock cultures were allowed to develop until the plates contained high numbers of gravid adults, but before food became limiting. Worms were then washed off the plates with K medium (53 mM NaCl, 32 mM KCl; 15) and eggs isolated by lysis with 2% NaOCl, 0.1 M KOH (14). Eggs from these ts strains appeared slightly more fragile than wild-type, so it was crucial to limit exposure to NaOCl (5-7 min total). After low-speed centrifugation (3000 x g for 3 min at room temperature), the egg pellet was washed several times with excess (50 ml) K medium. The final egg pellet was resuspended in 1 ml of K medium, a 1.0 µl aliquot was checked microscopically to determine egg numbers and integrity, and the remainder distributed among 10-20 NGM high-

peptone plates, usually using 6-well multiwell plates (Corning Inc, New York, USA). These were placed at 15, 20, 21 (two batches, one exposed and one control), 22 or 25°C for up to 9 days, until the hatched worms reached adulthood. RF exposure was carried out for 24 h (48 h for MT1514) at 21°C during the thermocritical phase (see details for each strain below). This temperature was selected for all of the ts strains because it leads to the development of roughly equal proportions of wild-type and mutant phenotypes under control (sham) conditions. Exposed and sham-exposed groups were placed in two identical copper transverse electromagnetic (TEM) cells held on the same shelf in a 21°C incubator, and an RF field (1.0 GHz, with 0.5 W input power; details in 3) was applied to only one of these. For the later studies shown in Table 1, a modified silver-plated TEM cell (10; see below) was used for RF exposure, while sham controls were housed in an unmodified copper cell. For the GE1076 strain, viable larvae (versus unhatched eggs and dead L1 larvae) were counted 24 h after the end of exposure, or after 48 h at 15°C. For all of the other strains, adults were washed off the plates with K medium and frozen down for later phenotype analysis. Thawed samples were aliquotted onto slides and coverslips added to prevent desiccation; all adult worms in each field were scored as wild-type or mutant until >200 had been scored. It was important to assess adult phenotypes within 2 days of the onset of egg-laying, so that they could not become confused with larvae of the F1 generation. Small larvae (<300 μm) were therefore ignored in the phenotype assessments, which used only the larger adults (>800 μm).

Statistical analysis: At least 6 data sets have been pooled for each of the figures presented here, apart from Table 1 where only 3 data sets are available. Each individual data set has been subjected to χ^2 analysis (numbers of mutant versus numbers of wild-type worms for 21°C sham versus 21°C RF-exposed conditions). ANOVA tests were also performed on the pooled data sets. For all strains except JK509, data generated by at least two different researchers have been used, but there is little sign of observer bias in identifying mutant phenotypes (for which strict criteria were established; see below). Where possible (for all adult phenotypes, but not for GE1076), frozen samples were blind-coded, so that the assessor did not know whether a given worm sample had been exposed or not.

Characteristics of the ts strains:

DH202 (b202)

The DH202 ts mutant affects the TRA-2 transmembrane receptor involved in sex determination. Worms are 98% wild-type (hermaphrodite, with eggs in the adult stage) at 15°C, but develop as

phenotypic males (without eggs and with a tail fan) at 25°C (16). Although TRA-2 is expressed in both sexes, its function is blocked in males through binding of the male-specific HER-1 ligand. For phenotype scoring, all worms showing blunt male tail structures (copulatory bursa and/or fan) were scored as mutant; most of these mutants also lacked eggs entirely. Wild-type *tra-2* activity is first required during late embryogenesis to establish hermaphrodite somatic development, therefore RF exposure was carried out for 24 h on freshly isolated eggs (which reached the L2 stage after 24 h).

JK509 (q231)

The JK509 ts mutant affects the GLP-1 transmembrane receptor involved in the decision between germ-line mitosis and meiosis (17). When GLP-1 is active (15°C), germ-line nuclei undergo several rounds of mitosis before entering meiosis, such that normal numbers of germ cells (both L4 sperm and adult eggs) are produced. Under restrictive conditions (25°C), germ-line nuclei enter directly into meiosis such that very few germ cells are produced; mutant adults therefore contain a few sperm but no eggs. At all temperatures, JK509 adults lacking eggs were scored as mutant. Wild-type *glp-1* function is required maternally during embryogenesis to establish dorso-ventral polarity, and again zygotically during the larval stages for germ-line mitosis versus meiosis (17). Worms hatched from JK509 eggs were grown up to the L2 stage at 21°C for 24 h and were then exposed to RF (or sham conditions) for the next 24 h, i.e. during the L3 stage which is thermocritical for germ-line mitosis.

MT1514 (n709)

The MT1514 ts mutant affects the LIN-39 homeodomain transcription factor. Mutants fail to develop a vulva, with the result that self-fertilised eggs hatch internally within the adults, producing an easily scored "bag-of-worms" phenotype. Unfortunately, MT1514 shows poor penetrance; 18% of worms show mutant characteristics under permissive conditions (15°C) while barely 40% do so at restrictive temperatures (25°C). The *lin-39* gene acts during embryogenesis to establish central regional identity (18), but also has a later role during L3 in vulva formation. Both early and mid-larval thermocritical periods were covered by exposing MT1514 worms for the first 48 h after egg plating. Only "bag-of-worms" adults were scored as positive for the mutant phenotype.

GE1076 (***e2123,*** also carrying the ***e678*** *lon-2* mutation)

The *e2123* ts mutation is a ts embryonic lethal allele affecting the PHA-1 nuclear transcription factor, which is required for normal development of the pharynx. At restrictive temperatures, most worms fail to hatch, and those few which do are unable to feed and therefore die as L1 larvae. This ts *e2123* mutation (combined with a non-ts *lon-2* mutation) allows the wild-type *pha-1* gene to be used as a selectable marker for generating transgenic strains on the mutant background (19). By growing eggs from injected worms at 25°C, only those which have acquired the wild-type *pha-1* gene (along

with the introduced transgene) will be able to survive, whilst non-transgenic progeny remain mutant for *pha-1* and perish at this restrictive temperature (19). We used the ts embryonic lethal phenotype of GE1076 as a second temperature-sensitive nuclear protein that might be affected by RF exposure. For this strain, late L4 larvae were exposed at 21°C to RF fields for 24 h, during which adults begin to lay eggs. Larval viability relative to dead eggs/larvae was assessed after 24 h, or after 48 h at 15°C

Bacterial culture and assay procedures: ML35 bacteria were grown to log phase (OD at 600 nm of 0.4) in 20 ml of LB broth, then centrifuged (4000 x g, 5 min) and the pelleted cells resuspended thoroughly in 40 ml of reaction mix (25 mM Tris-HCl pH 7.5, 125 mM NaCl, 2 mM $MgCl_2$, 12 mM β-mercaptoethanol) containing 0.05 mg ml^{-1} of the substrate 4-methylumbelliferyl-β-D-galacto-pyranoside (MUG). For reliable results, it was important to pre-incubate the reaction mix at the desired assay temperature, and to subdivide the bacterial suspension rapidly between 2 exposed and 2 sham-exposed 6-well plates (1 ml of bacterial suspension in reaction mix being added with swirling to each of the 24 wells). Exposures to RF (1.0 GHz, 0.5 W) fields were for 45 or 90 minutes at 35 or 37°C using a copper TEM cell as above. All exposures were started within 2 minutes of resuspending the bacteria. Sham controls were placed in the second TEM cell with no RF field (power off), positioned on the same incubator shelf. Four replicate 10 μl samples were then removed from each well and added to 200 μl of glycine-carbonate buffer (133 mM glycine, 83 mM Na_2CO_3 pH 10.7) previously placed in the wells of a black non-fluorescent microplate. The reaction product, 4-methylumbelliferone (4-MU, which is highly fluorescent in alkaline solution), was quantified on a Perkin-Elmer HTS7000 plate reader, calibrated against 4-MU standards across the range 0-1000 pM. Sham-sham controls for this experiment show some variability (up to 5% per run above or below the mean from several runs), but no overall pattern or trend. By contrast, exposed bacteria consistently show slightly higher β-galactosidase activity than do parallel sham controls. For obvious reasons, rapid processing of samples is essential for accurate results, and the 6 wells in each of the 4 plates within each run were set up and processed in exactly the same order. The order in which the 4 plates were processed was varied from run to run, but there was no detectable influence of sample processing order on the final outcome.

Characteristics of the exposure system: RF (microwave) exposures were performed in the transverse electromagnetic (TEM) cell described previously (3), using standard conditions of 1.0 GHz and 0.5 W input power (4, 5). Modelling of the Specific Absorbed Radiation (SAR) distribution for multiwell plates within this TEM cell suggests that the average SAR is around 15 mW kg^{-1}, with

maximal exposures of up to 40 mW kg^{-1} (averaged over a 1 g sample) confined to the edges (FDTD numerical modelling performed by M. Swicord and G. Bit-Babik, Motorola Research Laboratories, Florida, USA). These SARs are two orders of magnitude below those likely to cause detectable heating. Prior to and following exposure, both exposed and sham-exposed cultures were maintained at 21°C without RF irradiation. Careful monitoring of final sample temperatures with a sensitive microthermocouple (4) showed the temperature of exposed samples to be about 0.1°C warmer than that of sham-exposed samples. Traceable calibration of temperature parameters in our copper TEM cells by the National Physical Laboratory (NPL) suggested a slightly greater disparity, whereby exposed samples are ≤ 0.2°C warmer than sham controls as a result of power losses within the live cell (10). Since this slight heating appears sufficient to account for heat-shock reporter induction in transgenic *C. elegans* (10), we have repeated our phenotype-shift studies for two of the above strains (DH202, GE1076) by exposing worms or eggs to RF in a modified silver-plated TEM cell, in which the temperature differential between exposed and sham samples is reduced to ≤ 0.1°C (10).

RESULTS.

Following RF exposure at 1 GHz and 0.5 W, we observed a small but consistent (though non-significant) increase of 4-8%in β-galactosidase activity in exposed as compared to sham-exposed samples (Fig. 1B). However, there was no apparent difference between exposure temperatures (35 versus 37°C), nor between 45 and 90 min exposure periods. Comparisons between control β-galactosidase activities across the temperature range used in our ML35 work (35-39°C) shows an approximate doubling of enzyme activity for a 4°C rise in temperature (Fig. 1A). On this basis, a 6% increase in activity (the average seen across all RF exposures) could be explained by a temperature rise of around 0.25°C. This is quite plausible, given that there is known heating of around 0.1-0.2°C within the TEM cell used (3, 4, 10). Overall, Figure 1 suggests that RF fields do not significantly affect membrane fluidity (12). Similar studies with 50 Hz ELF electrical fields (at 1000 or 10,000 V m^{-1}; data not shown) tell a similar story; small (4-8%) increases in β-galactosidase activity are again observed, but there are no indications of temperature-, time- or dose-dependence in the data. We conclude that these probably result from heating artefacts caused by small temperature variations between exposed and sham positions. However, the ML35 membrane fluidity assay is probably not sensitive enough to pick up very small effects of RF or ELF exposure.

Turning to the *C. elegans* ts mutants, our initial aim was to test whether RF exposure might exert preferential effects on the conformation of thermolabile transmembrane proteins. As shown in

Figure 2, the DH202 ts *tra-2* mutant affecting sex determination is very strongly penetrant, with only 3% of eggs developing into males at 15°C as compared to 88% at 25°C. At the intermediate temperature of 21°C, 55% of eggs develop into mutant male worms (characterised by a tail fan and/or the absence of eggs). The prevalence of these mutant males increases dramatically to >78% in the case of eggs exposed to RF during the first 24 h of development at 21°C (since functional TRA-2 protein is required during late embryogenesis). RF irradiation of eggs apparently shifts the normal 21°C phenotype mix strongly towards the mutant end of the spectrum (Fig. 2). This effect of RF at 21°C is highly significant when compared against 21°C sham controls ($p < 0.001$ for each of 6 data sets). At the slightly lower intermediate temperature of 20°C, nearly 34% of worms show the mutant male phenotype – suggesting that the effect attributed to RF exposure would require quite substantial heating (≤ 0.5°C) in this instance. However, this remains an indirect inference.

To check whether this effect was unique to the DH202 strain, we tested a second ts mutant affecting a transmembrane protein – in this case the JK509 strain affecting the GLP-1 receptor required during larval development for germ-line mitosis. Again, this is a strongly penetrant temperature-sensitive mutation, with only 3% of worms showing the mutant phenotype at 15°C as compared with 94% at 25°C (Fig. 3). At the intermediate temperature of 21°C, some 48% of worms are mutant (i.e. carry no eggs), a proportion which increases to 65% following RF irradiation of developing larvae. At the lower intermediate temperature of 20°C, only 11% of worms fail to develop eggs (Fig. 3) – again suggesting that heating of the order of ≤ 0.5°C would be needed to achieve the same effect as RF.

To test the Kotnik and Miklavcik hypothesis (13), we also chose *C. elegans* ts mutants that affect genes encoding nuclear transcription factors:- specifically, MT1514 affecting *lin*-39 and GE1076 affecting *pha-1*. Unfortunately, the MT1514 ts mutant is poorly penetrant, with 18% of eggs developing into mutant vulva-less (bag-of-worms) adults at 15°C, as compared to only 39% at 25°C (Fig. 4). The intermediate temperature of 21°C gives 25% of mutants, but this percentage is only slightly increased to 28% following RF irradiation of eggs and larvae during their first 48 h of development (difference not significant in any of the 7 data sets analysed; $p > 0.05$). This longer exposure period was chosen to encompass both the embryonic requirement for LIN-39 function for establishing the central body region and the L3 requirement for LIN-39 in vulval development. The proportion of mutant phenotypes rises only gradually with increasing temperature (e.g. 22% at 20°C and 29% at 22°C, though even this difference does not reach statistical significance). Therefore no firm conclusions can be drawn from our work on MT1514.

Instead, we turned to the GE1076 ts mutant strain, used widely for *pha-1* selection of transgenic strains (19). The ts *pha-1* mutation (*e2123*) confers an embryonic lethal phenotype which is 100% lethal at 25°C, and which is almost as effective (over 99% lethal) even at 22°C (Fig. 5). GE1076 worms develop and reproduce more normally at 15°C. However, a substantial proportion of GE1076 eggs (averaging 38% in our hands) either fail to hatch or produce non-viable larvae; the corollary of this is that 62% of the eggs hatch into viable larvae that grow beyond the L1 stage. Inevitably, both non-viable eggs and larvae contribute to the embryo-lethal mutant score in Figure 5. The GE1076 strain shows a sharp temperature transition between wild-type and mutant phenotypes, since the prevalence of non-viable eggs at 20°C is 40% (i.e. 60% viable larvae), only slightly higher than at 15°C. At the intermediate temperature of 21°C, some 82% of the eggs are non-viable (18% viable escapers), but this increases to 92% following RF exposure of the eggs for 24 h at 21°C (8% escapers). This should be compared to 99% (1% escapers) at a higher temperature of 22°C with no RF. Despite variation in egg viability between runs, these figures are fairly consistent across 8 independent experiments, and the difference between RF and sham conditions was significant ($p < 0.001$) in every case. Nevertheless, an RF-induced increase of 10% in the prevalence of non-viable mutants could be explained in terms of an accompanying temperature increase of only 0.2- 0.5°C.

One crucial issue in these studies concerns the steepness of the transition from wild-type to mutant modes of development as the ambient temperature is raised. Such transitions are likely to be sigmoid in shape, although the evidence for MT1514 (Fig. 4) suggests that the slope can be quite shallow for some (poorly penetrant) mutations. However, this sigmoid transition could be very much steeper for strongly penetrant mutations (DH202, JK509 or GE1076) across the intermediate temperature range used here, such that very small temperature increases might cause major shifts in mutant prevalence. In view of our recent evidence suggesting that small temperature disparities (≤ 0.2°C) are sufficient to explain the induction of heat-shock reporter expression originally attributed to RF exposure (10), we have repeated our phenotype shift studies for 2 of these ts strains (DH202 and GE1076), using a modified silver-plated TEM cell for the RF exposures. In the modified cell, the temperature disparity between exposed and sham samples is reduced (≤ 0.1°C), as a result of minimising power losses in the live cell (10). As shown in Table 1, under these exposure conditions there is no longer any detectable shift in phenotype prevalence between sham and RF-exposed samples at 21°C for either of the strains studied ($p > 0.05$ from χ^2 tests in all 3 runs for each strain). Therefore, the phenotype shifts seen above (Figs. 2-5) are likely to result from very slight heating, as discussed below.

DISCUSSION.

A key issue raised by this study is the extent to which observed RF bioeffects could be thermal in origin (20). Although the RF dose received by worms – as estimated by numerical modelling (5-40 mW kg^{-1}; G. Bit-Babik and M. Swicord, Motorola Research Laboratories) and confirmed by direct field measurements at NPL (10) – is far too small to cause measurable microwave heating, this need not necessarily imply that the biological effects we have attributed to RF exposure (3-5) require a non-thermal explanation. For instance, it is possible that certain worm tissues (e.g. cuticle or eggs) may have unusual dielectric properties, causing localised "hotspots" where thermal effects might be significant. Among the ts mutant genes studied here, *lin-39* is expressed regionally in the central body, but *pha-1* is expressed in the pharynx only, while both *tra-2* and *glp-1* genes are expressed more widely – the latter mainly in germ-line tissue (which largely fills the pseudocoelomic space). Based on the thermal calibration of our copper exposure cell conducted at NPL (10), a temperature rise of ≤ 0.2°C would be expected in RF-exposed as compared to sham samples under standard exposure conditions (1 GHz, 0.5 W). In fact, this is only slightly greater than the 0.1°C disparity noted previously between the temperatures of RF-exposed and sham samples, as measured with a sensitive microthermocouple immediately after a 20-hour exposure at 750 MHz and 0.5 W (4). Is this heating sufficient to explain the observations reported here and previously (3-5)? Fortunately, this question can be addressed by conducting RF exposures in a modified silver-plated version of our TEM cell (10) that reduces the temperature disparity between exposed and sham samples to ≤ 0.1°C.

Certainly the small RF-induced increases in β-galactosidase activity in ML35 bacterial cells are best explained as a thermal artefact. This is supported by the lack of time- or temperature-dependence in the data set (Fig. 1B). 50 Hz ELF electrical fields produce similarly small effects, and in this case there is no sign of dose-dependence either (10,000 versus 1,000 V m^{-1}). Turning to the ts *C. elegans* mutants, it is not worth further discussing the MT1514 mutant because of poor penetrance and a lack of statistically significant differences in mutant prevalence. For a second ts mutant affecting a nuclear protein (the PHA-1 transcription factor in GE1076 worms; Fig. 5), there seems to be a clear and significant 10% increase in mutant prevalence following RF exposure, but this disappears (Table 1) when RF exposures are conducted in the modified cell to minimise thermal disparities (10). This strain also shows a very sharp transition from larval viability (~60% at 15°C and 20°C) to lethality (> 99% at 22°C and 25°C). On both counts, a thermal explanation (≤ 0.2°C) seems sufficient to account for the apparent effects of RF exposure on phenotype prevalence in the copper cell (Fig. 5).

By contrast, both JK509 and DH202 strains show a higher incidence of the mutant phenotype at 20°C as compared to 15°C (11% for JK509 in Fig. 3; 34% for DH202 in Fig. 2), and the increased prevalence of the mutant phenotype attributable to RF exposure at 21°C in the copper cell is also somewhat greater (17% for JK509 in Fig. 3; 23% for DH202 in Fig. 2) than in the case of GE1076. However, for DH202 at least, this apparent effect of RF also disappears when exposures are conducted in the modified cell (Table 1) – once again suggesting that the differences attributed to RF at 21°C (Fig. 2) most probably result from a slight temperature disparity (≤ 0.2°C) between exposed and sham samples when using the original copper cell for RF exposure. This in turn suggests that the (sigmoid) transition from wild-type to mutant development must be particularly steep for strain DH202 around 21°C. We have not yet tested strain JK509 using the modified cell for RF exposures, but it is unlikely that the smaller changes seen here (Fig. 3) would require a non-thermal explanation.

At first sight, the findings reported in Figures 2-5 provide tentative experimental support for the model proposed by Kotnik and Miklavcik (13), since the effects of RF on the prevalence of mutant phenotypes seem greater for ts mutations affecting transmembrane (JK509, DH202) as compared to nuclear (MT1514, GE1076) proteins. However, this cannot be sustained in the light of Table 1, where RF exposure has no discernible effect on mutant prevalence for either DH202 or GE1076 strains at 21°C, under conditions where the temperature disparity between exposed and sham conditions is reduced by ~50%. The contrast between Table 1 and the earlier data (Figs. 2-5) implies that very slight heating can substantially increase mutant prevalence, but that RF has no independent effect. It could be argued that genuine RF bioeffects might synergise with those of heat. However, even this seems unlikely in the light of thermal calibrations of both our original copper and modified silver-plated TEM cells at NPL (10), which show that sample heating during RF exposure is reduced (from ≤ 0.2°C to ≤ 0.1°C) but not entirely eliminated in the modified cell. Although worms are exposed to essentially the same RF fields in the modified cell as in the original copper cell, they no longer show any detectable effect of RF exposure relative to shams, either in terms of induced heat-shock reporter expression (10) or increased prevalence of ts mutant phenotypes (this paper).

Equally, our findings cannot disprove the Kotnik and Miklavcik model (13), except in the rather narrow context of protein denaturation for highly thermolabile proteins. Other forms of RF action at the plasma membrane cannot be excluded, although our work with ML35 bacteria (this paper) seems to rule out any major effect on membrane permeability. A more likely target might be the functions of ion channels, and here the use of ts strains such as DH202 could provide an important internal

control to demonstrate the absence of confounding thermal effects. However, our focus in this paper was to determine whether weak RF fields could have measurable effects on protein conformation. We have previously reported a small but apparently significant enhancement of protein aggregation by RF in concentrated solutions of bovine serum albumin (BSA; 21). When this was repeated using the modified TEM cell for exposure, the effect of RF on aggregation was reduced in magnitude and no longer reached statistical significance. Moreover, this remaining effect showed no evidence of dose dependence when plotted well by well against the known SAR distribution, suggesting that RF exposure was not the causative agent. The small remaining temperature disparity of ≤ 0.1°C is probably sufficient to explain any residual difference between exposed and sham samples (B. Smith, unpublished data). Thus three different lines of evidence – from heat-shock reporter expression (10), BSA aggregation, and ts mutant prevalence (this paper) – all now point towards the same conclusion:- namely, that weak RF fields do not measurably affect protein conformation.

Our findings carry an important corollary, however, since the effects described here and previously (3-5, 10, 21) clearly result from very slight heating (0.1-0.2°C) rather than exposure to RF fields. This demonstrates that biological processes can be very finely attuned to the ambient temperature, and that slight heating cannot be dismissed as a plausible explanation for purported EMF bioeffects. That said, it would be inappropriate to extrapolate directly from a poikilothermic system at the upper end of its temperature tolerance range (*C. elegans* at 20-25°C) to a homiothermic mammal such as mouse or human, where the normal core body temperature can be exceeded by 3-5°C during a fever.

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FIGURE LEGENDS.

Figure 1. Effects of RF exposure on inner membrane leakage in ML35 *E. coli* cells.

Part A. Temperature dependence of substrate leakage into ML35 cells.
Rates of MUG substrate leakage into ML35 cells were measured as described in Methods at 3 different temperatures (35, 37 and 39°C) over 45 min (dashed line, squares) or 90 min (solid line, triangles) time periods. Mean and SD (from 4 replicates per point) are shown as picomoles of MU product per mg protein.

Part B. Effect of RF on substrate leakage into ML35 cells at 35 and 37°C.
ML35 cells were exposed to RF fields (1.0 GHz, 0.5 W) at either 35°C (columns 1-4) or 37°C (columns 5-8) or sham-exposed at the same temperature, in both cases for either 45 minutes (columns 1, 2, 5 and 6) or 90 min (columns 3, 4, 7 and 8). In all cases, β-galactosidase activities for RF samples have been normalised against the corresponding mean sham values as 100%. Means and standard deviations from 4-8 replicates are shown for all columns. From left to right:- column 1, sham 45 min at 35°C; column 2, RF-exposed 45 min at 35°C; column 3, sham 90 min at 35°C; column 4, RF-exposed 90 min at 35°C; column 5, sham 45 min at 37°C; column 6, RF-exposed 45 min at 37°C; column 7, sham 90 min at 37°C; column 8, RF-exposed 90 min at 37°C.

Figure 2. Effect of temperature and RF on phenotype prevalence in strain DH202.

The prevalence of mutant male phenotypes among adults derived from DH202 eggs was assessed at various temperatures (15, 20, 21 and 25°C), comparing RF-exposed (1.0 GHz and 0.5 W for the first 24 h of development) against sham-exposed eggs at the intermediate temperature of 21°C. Phenotype assessment and sample blinding was performed as described in Methods. Each column shows the mean and SEM derived from 4 (20°C only) or 6 (all other columns) independent runs, each involving phenotype assessment of at least 500 adult worms.

Figure 3. Effect of temperature and RF on phenotype prevalence in strain JK509.

The prevalence of mutant phenotypes (lacking eggs) among adults derived from JK509 eggs was assessed at various temperatures (15, 20, 21 and 25°C), comparing RF-exposed (1.0 GHz and 0.5 W during the second 24 h of development) against sham-exposed larvae at the intermediate temperature of 21°C. Phenotype assessment and sample blinding was performed as described in Methods. Each column shows the mean and SEM derived from 3 (20°C only) or 6 (all other columns) independent runs, each involving phenotype assessment of at least 500 adult worms.

Figure 4. Effect of temperature and RF on phenotype prevalence in strain MT1514.

The prevalence of mutant "bag-of-worms" phenotypes among adults derived from MT1514 eggs was assessed at various temperatures (15, 20, 21, 22 and 25°C), comparing RF-exposed (1.0 GHz and 0.5 W for the first 48 h of development) against sham-exposed eggs/larvae at the intermediate temperature of 21°C. Phenotype assessment and sample blinding was performed as described in Methods. Each column shows the mean and SEM derived from 4 (20 and 22°C only) or 7 (all other columns) independent runs, each involving phenotype assessment of >500 adult worms.

Figure 5. Effect of temperature and RF on phenotype prevalence in strain GE1076.

The prevalence of viable larval escapers derived from GE1076 eggs was assessed at various temperatures (15, 20, 21, 22 and 25°C), comparing RF-exposed (1.0 GHz and 0.5 W for the first 24 h of development) against sham-exposed eggs at the intermediate temperature of 21°C. All eggs and dead L1 larvae were counted as non-viable, and the numbers of viable escapers were assessed either 48 h (15°C only) or 24+ h (all other temperatures) after egg isolation. Each column shows the mean and SEM derived from 5 (20 and 22°C only) or 10 (all other columns) independent runs, each involving at least 500 worm eggs and larvae. Note that blinding of samples was impracticable in this instance, because live larvae had to be distinguished from dead larvae and unhatched eggs (all counted as non-viable) under the microscope during a brief interval after exposure.

Table 1. Effects of RF exposure with minimal heating on mutant phenotype prevalence in DH202 and GE1076 strains.

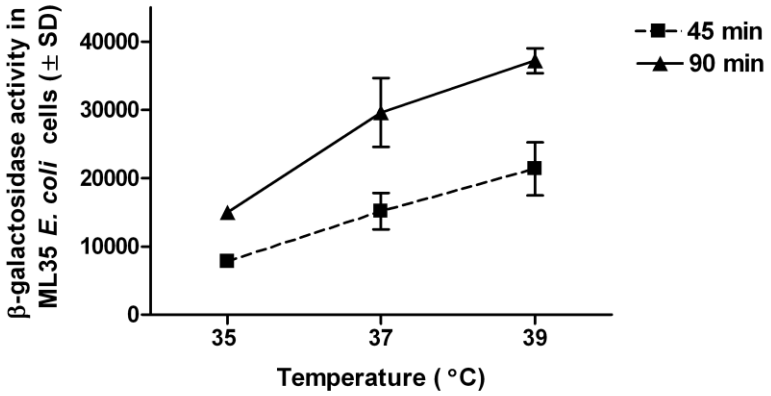
Temperature and condition	Percentage of mutant (male) adult worms for strain DH202	Mean number of viable progeny (escapers) per adult worm for strain GE1076
15°C	**3.2 ± 0.8**	**196 ± 18**
21°C (sham in copper cell)	**59.5 ± 2.6**	**87 ± 8**
21°C (RF-exposed in modified cell)	**60.3 ± 2.3**	**91 ± 7**
25°C	**83.3 ± 6.9**	**4 ± 2**

The experiments shown in Figures 2 (DH202) and 5 (GE1076) were essentially repeated using the modified silver-plated TEM cell for RF exposures (with sham controls in a copper TEM cell). The tabulated values are means ± SEMs derived from 3 repeat runs for each strain. For DH202, the percentage of adults showing unambiguously male (blunt) tail structures was scored for > 200 worms per run (note that this strict criterion somewhat reduces the prevalence of mutants at 25°C). For GE1076, the number of viable offspring observed was divided by the number of adult worms present, so as to calculate a mean number of progeny per adult (i.e. the converse of Figure 5). For both strains, χ^2 tests applied to the raw data return a p value > 0.05 in each of the 3 runs (comparing exposed against sham in all cases). Overall, RF exposure has no effect on mutant prevalence.

Figure 1.

A

Temperature dependence of substrate leakage into ML35 cells



B

RF field (1.0 GHz, 0.5 W) at 35 and 37 °C

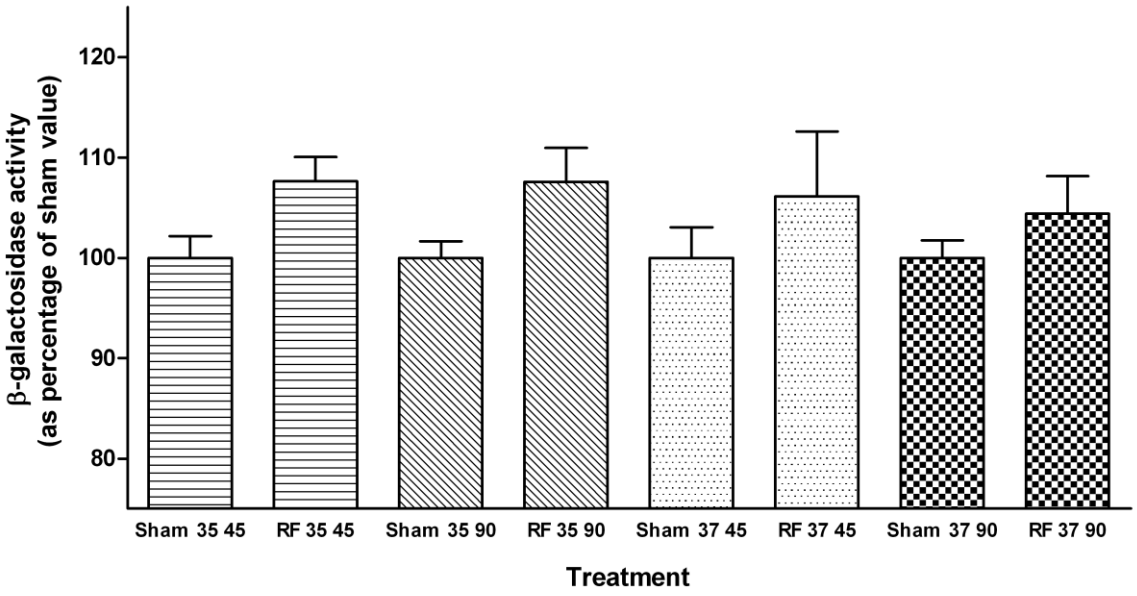


Figure 2.

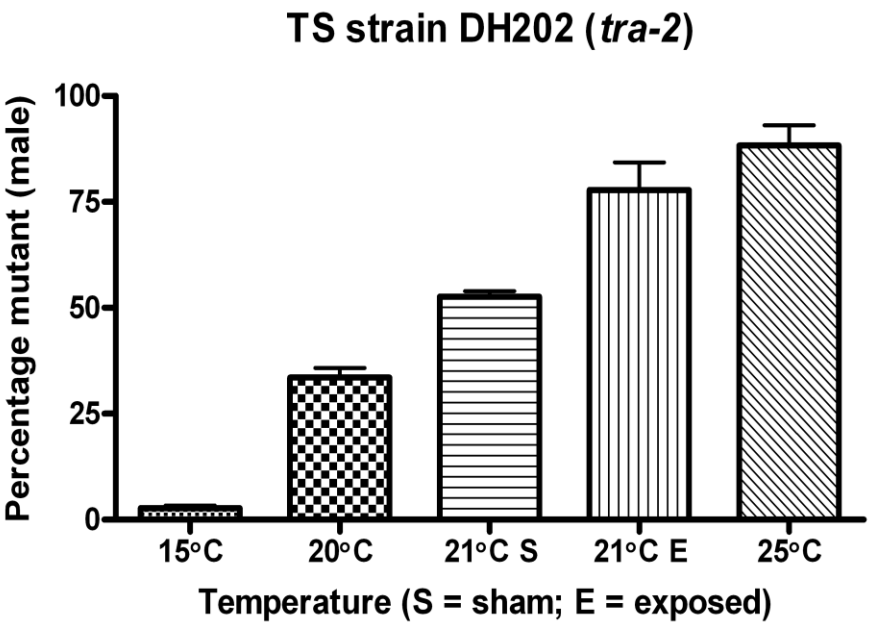


Figure 3.

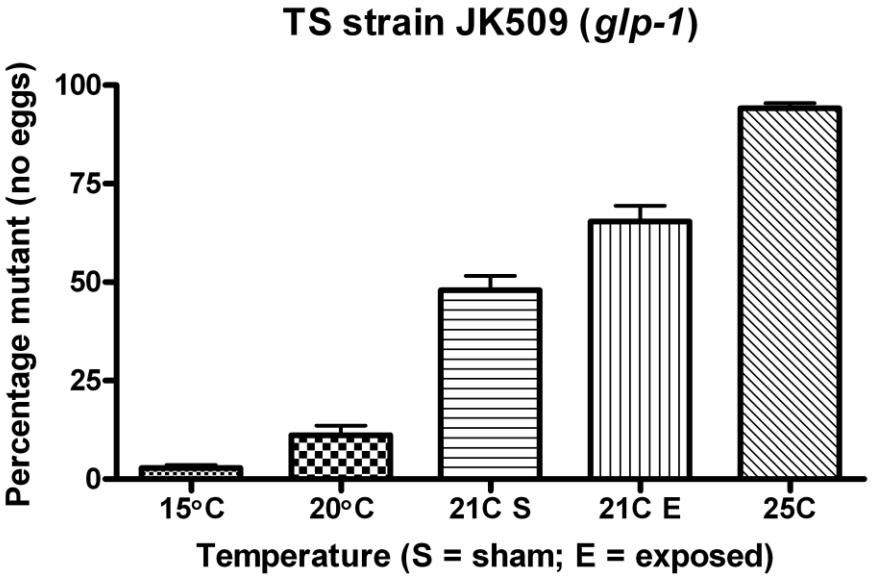


Figure 4.

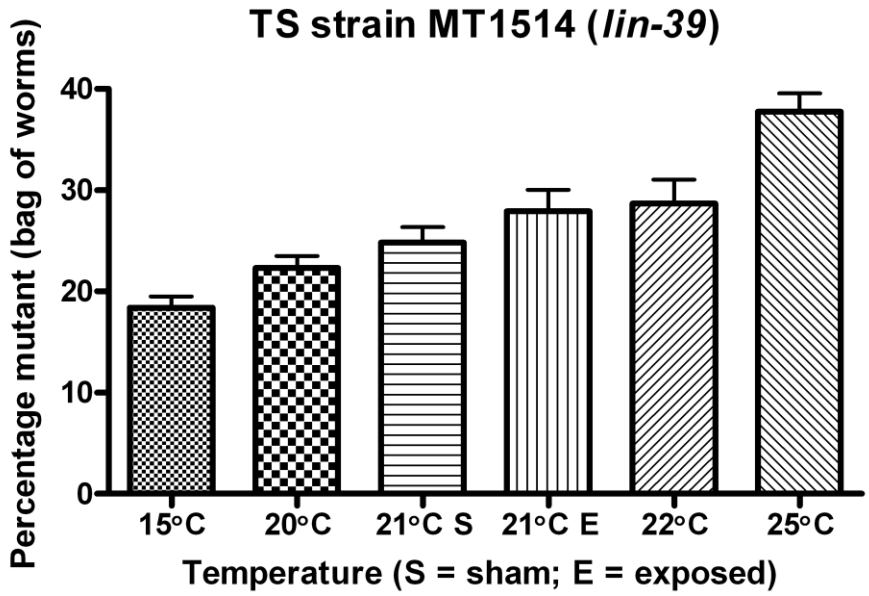


Figure 5.