

Phylogenetic assignment and mechanism of action of a crop growth promoting *Rhizobium radiobacter* strain used as a biofertiliser on graminaceous crops in Russia

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Abstract The taxonomic position of “*Agrobacterium radiobacter* strain 204,” used in Russia as a cereal crop growth promoting inoculant, was derived by a polyphasic approach. The phenotypic analyses gave very similar biochemical profiles for strain 204, *Rhizobium radiobacter* NCIMB 9042 (formerly the *A. radiobacter* type strain) and *R. radiobacter* NCIMB 13307 (formerly the *Agrobacterium tumefaciens* type strain). High percentage similarities, above the species separation level, were observed between the 16S rRNA, *fusA* and *rpoB* housekeeping gene sequences of these three strains, and the genomic DNA–DNA hybridisation of strain 204 against the type strain of *R. radiobacter* NCIMB 9042 was over 70%. Strain 204 is not phytopathogenic and it does not fix atmospheric N₂ or form a physical association with

the roots of barley. Strain 204 culture and culture supernatant stimulated the rate of mobilisation of seed reserves of barley in darkness and promoted its shoot growth in the light. Gibberellic acid (GA) concentration was 1.3 μM but indole acetic acid was undetectable (< 50 nM) in cultures of strain 204. It is concluded that strain 204 is phenotypically and genotypically very similar to the current *R. radiobacter* type strain and that the mechanism of its effect on growth of cereals is via the production of plant growth promoting substances. GA is likely to play an important role in the strain 204 stimulation of early growth of barley.

Keywords Cereals · Crop growth promotion · Gibberellic acid · N₂ fixation · Phylogenetic assignment · *Rhizobium radiobacter*

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Introduction

There is strong evidence that some commercially important tropical grasses e.g., sugar cane and wetland rice can obtain a significant amount of their nitrogen (N) requirements from naturally occurring associated N₂ fixing soil bacteria (James 2000). This is not the case with commercially important temperate graminaceous species and considerable research has been carried out in attempts to increase the yield of temperate cereals by inoculation with N₂ fixing bacteria. In many studies, inoculation of temperate cereals with *Azospirillum* in particular, but also several other plant growth promoting bacteria (PGPB), resulted in increased plant/crop growth and yield (Andrews et al. 2003). However, there is general agreement that these growth responses were not due to N₂ fixation by the bacterium, but were primarily related to the bacterial production of plant growth substances, which caused changes in root morphology and physiology that resulted in increased nutrient and water uptake from the soil (James 2000; Andrews et al. 2003; Mantelin and Touraine 2004).

Reports of work carried out in Russia indicate that, at a soil pH above 5.5 and with adequate soil water, inoculation of wheat and barley with a bacterial strain, isolated in 1983 from the rhizosphere of a rice crop growing in the USSR and initially identified as *Agrobacterium radiobacter* (catalogued as *A. radiobacter* strain 204) on the basis of phenotypic properties, can result in crop yield increases equivalent to that obtained by the application of 30 kg N/ha (Bairamov et al. 2001; Zavalin et al. 2001). Generally, inoculation had no effect on yield in treatments where 30 kg N/ha was applied. *A. radiobacter* strain 204 was found to be non-pathogenic and non-toxicogenic (Omel'ianets et al. 1992) and it was made available to farmers as a biofertiliser, with the name Rhizoagrin. The bacterium was believed to be able to fix atmospheric N₂ on the basis of the results of acetylene reduction assays performed in Russia after its isolation and it was assumed that the effect of the bacterium on cereal growth was primarily due to N₂ fixation (Bairamov et al. 2001). However, although it was proposed that *Agrobacterium* should be amalgamated with

Rhizobium (Young et al. 2001; but cf Farrand et al. 2003), at this time there are no published reports of naturally occurring *Agrobacterium* spp. with symbiotic plasmids that can fix atmospheric N₂ in a symbiosis with a plant. Also, although *Agrobacterium* spp. have been isolated from the nodules of several tropical legumes, to date, none of these have been shown to be capable of nodulating the host plant (de Lajudie et al. 1999; Mhamdi et al. 2005). Controversially, it was suggested by Kanvinde and Sastry (1990), that *A. tumefaciens* strains B6, C58 and NT1 can fix atmospheric N₂ using a nitrogenase encoded by a *nif* gene located on the chromosome, rather than one located on a symbiotic plasmid. However, no *nif*-like gene was discovered on the chromosome of *A. tumefaciens* strain C58 when the genome sequence of that bacterium was assembled. Nevertheless, recently, the coexistence of symbiosis and pathogenicity-determining genes has been shown to occur in strains of *Rhizobium rhizogenes* which enables them to induce nodules or tumours depending on plant species (Velázquez et al. 2005).

In this study, phenotypic and genotypic characterisation techniques were employed to determine if the bacterium utilised in Rhizoagrin biofertiliser, in Russia, is indeed a strain of *Agrobacterium radiobacter* (hereafter, termed *Rhizobium radiobacter* after Young et al. 2001). Also, the ability of the bacterium to fix atmospheric N₂ and produce the plant hormones indole acetic acid (IAA) and gibberellic acid (GA), and the effects of complete and supernatant bacterial culture medium on growth of barley were assessed to gain understanding of the mechanism of the growth effect of the bacterium on cereals.

Materials and methods

Bacterial culture conditions, growth media and phenotypic testing

R. radiobacter strain 204 (hereafter referred to as strain 204), *R. radiobacter* NCIMB 9042 (or ATCC 19358, formerly *A. radiobacter* type strain) and *R. radiobacter* NCIMB 13307 (or ATCC

23308, formerly the *A. tumefaciens* type strain) were routinely grown in yeast mannitol broth (YMB, Vincent 1970). Antibiotic sensitivity testing and substrate utilisation testing in MOPS-salts medium were carried out on the three strains as previously described (Humphry et al. 2001). API 20NE and API ZYM test strips were also set up for these strains, according to their operating instructions.

Sequencing of the 16S rRNA, *rpoB* and *fusA* genes

Genomic DNA was extracted from strain 204 and the 16S rRNA gene sequence was amplified. The 16S rRNA primers used to obtain the initial PCR product were identical to the *Escherichia coli* 16S rRNA gene sequence, between positions 8 and 27 (in the forward position), and 1,509 to 1,491 (in the reverse position). These primers were 5'-AGA GTT TGA TCC TGG CTC AG-3' and 5'-GGH TAC CTT GTT ACG ACT T-3', respectively. The resulting 16S rRNA genes, for the bacterial isolate were cloned into pGEM-T plasmid (Promega) and sequenced (Lark Technologies Inc.). The methods used to extract the genomic DNA, and amplify and clone the 16S rRNA genes were as previously described (Humphry et al. 2001). The two plasmid inserts were sequenced completely, using a DNA sequencer. Initially, this was done with the M13 forward and reverse primers for the pGEM-T plasmid, and subsequently oligonucleotide primers were built onto the ends of the resulting sequences.

Amplification of the *rpoB* and *fusA* genes from strain 204 and the type strains shown in Fig. 1 were performed using the primer sequences described by Santos and Ochman (2004). The amplified PCR products were subsequently cloned and sequenced according to the methods previously described (Santos and Ochman 2004).

Sequence analysis

The 16S rRNA gene sequence from strain 204 was aligned with six sequences obtained from GenBank database (Benson et al. 1998), accession numbers are shown on the dendrogram. The

sequences were aligned using the CLUSTAL method in MEGALIGN program (DNASar) and all columns containing non-base characters were removed. This reduced the length of the sequences to 1,324 bases. The *rpoB* and *fusA* sequences generated during this study were treated

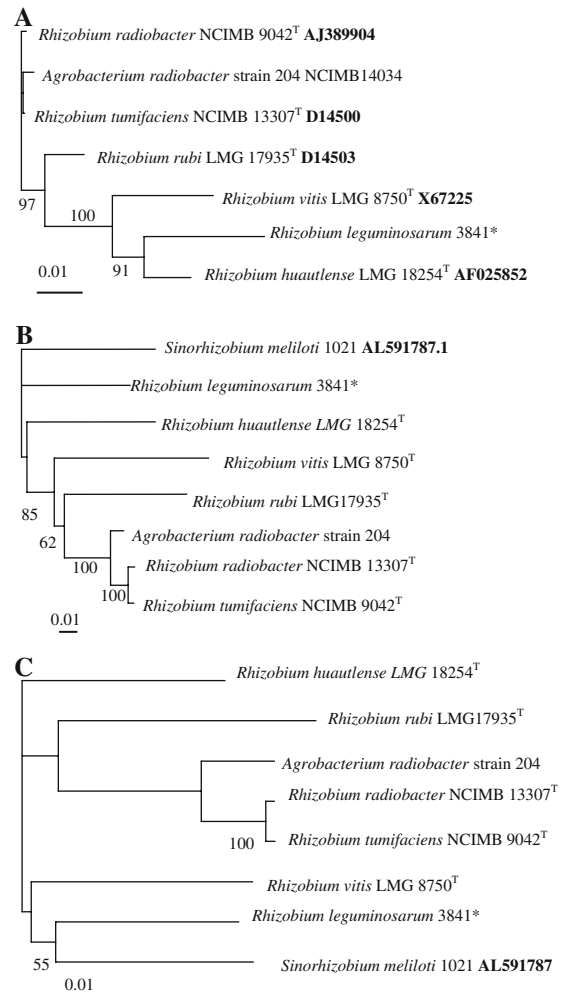


Fig. 1 Unrooted phylogenetic dendrograms based upon (A) 16S rRNA gene sequences, (B) *fusA* sequences and (C) *rpoB* sequences. The comparisons were made using the Jukes and Cantor algorithm and the Neighbor-joining method, bootstrap confidence percentages were also calculated from 1,000 replicate trees. These percentages were shown on the nodes if they occurred in more than 50% of the trees. The scale bar represents the number of nucleotide substitutions per 100 nucleotides. Numbers in bold are accession numbers for the GenBank database and T denotes the type strain of the species. *Rhizobium leguminosarum* 3841 is currently undergoing genome sequencing, sequences marked * are available from http://www.sanger.ac.uk/Projects/R_leguminosarum

in the same way, the resulting *fusA* and *rpoB* sequences were 751 and 714 bases in length, respectively. The programs used for phylogenetic analysis included SEQBOOT, DNADIST, NEIGHBOR, CONSENSE (PHYLIP 3.5c package; Felsenstein 1993) and TREEVIEW (Page 1996). Computerised phylogenetic analysis was done as described by Humphry et al. (2001).

DNA–DNA hybridisations

DNA–DNA hybridisations were carried out as described previously (Humphry et al. 2001). Briefly, the genomic DNA of strain 204 was extracted using a microcentrifuge separation column (Qiagen). The DNA was isolated by chromatography, then DNA–DNA hybridisation was performed on a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Finally, renaturation rates were computed using the TRANSFER.BAS programme.

Phytopathogenic testing

Crown gall production tests were performed on potato tuber tissue culture, as described by Anand and Heberlein (1997). Testing on young tobacco and tomato plants was done by creating wounds on their stems and inoculating them with strain 204 cultures as instructed by Moore et al. (2001). The PCR methods used to amplify the characteristic VirD2 and *ipt* gene portions found in functional “Agrobacterial” Ti and Ri plasmids were those described by Haas et al. (1995).

Growth on N-free media

Strain 204 was tested for diazotrophy using the following semi-solid N-free media that are semi-selective for those bacteria noted in brackets, these organisms were also included as positive controls: NFb (*Azospirillum brasilense*, *A. lipoferum*; Tarrand et al. 1978), JNFb (*Herbaspirillum* spp.; Olivares et al. 1996), JVM (*Burkholderia* “*tropicalis*”; Baldani et al. 2000), LG1 (*Azospirillum amazonense*; Magalhães et al. 1983) and LG1-P (*Gluconacetobacter diazotrophicus*; Reis et al. 1994). Firstly, growth and

pellicle formation in the various semi-solid media (Döbereiner 1992) were examined at 1–5 days after inoculation, and then nitrogenase activity was determined using the acetylene reduction assay according to Burris (1972). Briefly, 10% acetylene was injected into the head space of the culture flasks, and the concentration of ethylene in the head space gas was determined by gas chromatography after 1 h of incubation at 30°C. Uninoculated flasks were used as controls to detect background ethylene. In addition to the semi-solid media, BG11, a liquid N-free medium devised to test *Azotobacter* spp. for nitrogenase activity was also used as described by Rowell et al. (1998).

NifH detection

PCR to amplify *nifH* products was done according to the methods of Eardly et al. (1992) and Haukka et al. (1998), with primer pairs *nifH*-1/*nifH*-2 and *nifH*-1/*nifH*-5. However, a wider range of annealing temperatures (37°C, 41°C, 45°C, 49°C, 53°C and 56°C) and MgCl₂ concentrations (2 mM, 3 mM and 4 mM), were also tried. A positive control (*Rhizobium leguminosarum* 3841) was successfully used to ensure the PCR methodology did amplify the *nifH* gene using the primers pairs. Southern blotting was employed against genomic DNA using partial *Rhizobium leguminosarum* ATCC 10004, *Mesorhizobium loti* LMG 6125 and *Bradyrhizobium japonicum* USDA 110 *nifH* gene sequences as described by Rosado et al. (1998).

Effect of strain 204 culture or culture supernatant on seed germination and growth of barley

Two experiments were carried out on barley cv Maris Otter to assess if the strain 204 effect on cereal growth is due to the production of plant growth substances. In Experiment 1, the impact of strain 204 culture or culture supernatant on seed germination and seedling growth was tested. Seeds, selected on the basis of weight (54–55 mg), were germinated in Petri dishes on 10 sterile Whatman No. 1 filter papers, moistened with 10 ml of sterile distilled water, YMB, strain 204

culture ($OD_{660\text{ nm}} 1.0$) or culture supernatant obtained by centrifugation (15,000 rpm, 20 min, 4°C). Incubation was in the dark at 25°C. Two seeds were germinated per Petri dish and each treatment was replicated five times. Shoot and root length, and the number of root branches were determined 8 days after sowing. The experiment was repeated.

The longer term effects of strain 204 culture and culture supernatant on shoot growth were determined in Experiment 2. Barley seeds were sown in 0.5 l pots containing equal volumes of vermiculite and perlite. Four seeds were sown per pot, but seedlings were thinned to two per pot after emergence. Every 2 days, the pots were flushed through with a basal nutrient solution (Andrews et al. 1989) containing 2 mM N as KNO_3 in a 1:1 (v/v) mixture with YMB, bacterial culture or bacterial culture supernatant (prepared as described for experiment 1). The initial and repeat Experiment 2 were carried out between 10 April–20 May and 17 April–27 May 2004, respectively, in a glass-house under natural daylight at the University of Sunderland. The temperature ranged between 15°C and 28°C. At harvest, shoot dry weight was determined.

Quantitation of IAA and GA concentrations in culture supernatants

The concentration of IAA was determined in supernatant of six separate cultures of strain 204 using an IAA immunoassay detection kit as described by the manufacturers (Sigma-Chemie). GA concentration in supernatant of six separate cultures was determined using a Shimadzu HPLC system consisting of an LC-6A pump, a SPD-6AV variable wavelength UV/Vis. detector a CR5-A integrator and a Phenomenex Columbus 3 C18 100×2.0 mm column. A Nicolet-Evolution 300BB UV scanner (Dyson Instruments) was used to obtain the appropriate wavelength for gibberellic acid (Sigma) detection. Injections into the HPLC were made manually using 2.5 ml syringe and a Rheodyne 7125 injection valve (Anachem) fitted with a 2 ml loop. The mobile phase was a 20:80 (v/v) mix of methanol and 0.05 M perchloric acid (pH 2.7). For standards

and strain 204 cultures, 20 ml samples were acidified with 0.05 M perchloric acid, extracted into 4×10 ml of ethyl acetate, evaporated to dryness and reconstituted with 10% methanol in water.

Statistical analysis

The two experiments on barley were of completely randomised design. In both cases, data for the initial and repeat experiment were pooled for statistical analysis and presentation using one-way analysis of variance with growth medium as the variable. Variability quoted in the text is the standard error.

Results and discussion

The bacterial strain was putatively identified in Russia as *A. radiobacter* on the basis of the phenotypic classification outlined in Conn (1942). In the present study, the bacterium utilised maltose, D-mannitol, inositol, sucrose, trehalose, raffinose, xylose, glycerol, glucose, fructose, lactose and galactose, but not lactate or acetate as growth substrates. It had the following enzyme activities; urease, alkaline phosphatase, acid phosphatase, esterase, leucine and valine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase and it hydrolysed aesculin. There was no lipase, cysteine arylamidase, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -mannosidase, α -fucosidase, arginine dihydrolase activities or gelatin hydrolysis. Glucose acidification did not occur and indole was not produced from tryptophan. The bacterium was sensitive to the following antibiotics; gentamycin, fusidic acid, tetracycline, novobiocin, ampicillin and colistin sulphate, but it was not sensitive to penicillin, clindamycin, erythromycin, trimethoprim, sulphamethoxazole, chloramphenicol, methicillin, streptomycin, cephalothin, sulphatriad (containing sulphathiazole, sulphadiazine and sulphamerazine) and cotrimoxazole. These biochemical properties were identical to those obtained for *R. radiobacter* NCIMB 9042 and *R. radiobacter* NCIMB 13307.

Table 1 Differentiating biochemical tests and molecular characterisation of *R. radiobacter* strain 204 compared to *R. radiobacter* NCIMB 13307 and *R. radiobacter* NCIMB 9042

	<i>R. radiobacter</i> strain 204	NCIMB 13307	NCIMB 9042
<i>Biochemical test</i>			
β -Galactosidase	–	–	+
<i>Antibiotic disk</i>			
Fusidic acid (10 μ g)	+	–	+
Chloramphenicol (25 μ g)	–	+	+
<i>DNA similarities</i>			
16S rRNA similarity to <i>R. radiobacter</i> strain 204 (%)	NA	99	99
Genomic DNA homology to <i>R. radiobacter</i> strain 204 (%)	NA	74.5	67.8

Key: + Indicates enzyme activity or presence of a zone of inhibition (ZOI) greater than 2 mm and – indicates no enzyme activity or no ZOI present

The differentiating characteristics between these three bacteria are presented in Table 1.

This study demonstrated that the 16S rRNA gene sequence of strain 204 was 99% identical (1419/1423) to the type strain of *R. radiobacter* NCIMB 9042 and 99% identical (1434/1440) to that of *R. radiobacter* NCIMB 13307. 16S rRNA gene sequences analysis results are sometimes inaccurate because of lateral gene transfer, therefore, two conserved housekeeping genes, *rpoB* and *fusA*, were also sequenced. These genes gave similar phylogenetic similarities for the test strains to those produced by the 16S rRNA gene sequences. The three phylogenetic trees produced are shown in Fig. 1A–C. In each, strain 204 clusters with NCIMB 13307 and NCIMB 9042 supporting the designation of strain 204 as a strain of *R. radiobacter*. Genomic DNA homology was 74.5% between strain 204 and *R. radiobacter* NCIMB 13307, and 67.8% between strain 204 and NCIMB 9042 (Table 1), which confirms it is *R. radiobacter* if one uses the threshold value of 70% DNA–DNA homology as the requirement for a bacterial genomospecies (Wayne et al. 1987). Therefore, strain 204 is phenotypically and genotypically very similar to the current *R. radiobacter* type strain and the former type strain of *A. tumefaciens* (now *R. radiobacter*).

Strain 204 did not produce crown gall in potato tuber tissue culture or stems of tobacco or tomato. Also, it did not have the highly conserved *VirD2* or *ipt* gene portions which are found in *Ti* and *Ri* plasmids present in phytopathogenic “*Agrobacterium*” strains. These results are in

agreement with previous reports that strain 204 is non-pathogenic (Omel’ianets et al. 1992).

Having established the phylogenetic status of strain 204, the second aim of this study was to ascertain the mechanism of its growth effect on cereals. Firstly, it was assessed if strain 204 was an associative diazotroph that increased cereal growth by providing additional N to the plants via N_2 fixation. To date, there has been only one report of naturally occurring strains of *A. radiobacter* or *A. tumefaciens* that could fix N_2 (Kavinde and Sastry 1990) and there are some concerns about its accuracy. In order to resolve whether strain 204 is diazotrophic, as asserted by Bairamov et al. (2001), it was tested in a range of N-free growth media. Growth was observed in all the N-free media, however, growth was slow and very limited compared to the positive controls, suggesting that the bacterium was scavenging trace N from the media rather than fixing atmospheric N_2 . This was confirmed by acetylene reduction assays, that showed no significant production of ethylene above any of the negative controls in the media tested. In addition, studies to identify the presence of any *nifH* genes in the organism were unsuccessful, although it is acknowledged that the primers were based on *nifH* genes from sym plasmids. No PCR bands were amplified under any of the PCR conditions used that were close to the size of known *nifH* products. Moreover, no hybridisation of the *nifH* probes to genomic DNA under appropriately stringent conditions was observed. In addition, both light and electron microscopy studies carried

Table 2 Germination, seedling growth in the dark and shoot growth in the light of barley when treated with sterile water, YMB or *R. radiobacter* strain 204 culture or culture supernatant

Treatment	Germination/seedling growth, 8 day incubation, room temperature in dark			Shoot growth, 40 day incubation 15–28°C in light Shoot dry wt. (g)
	Shoot length (mm)	Root length (mm)	Root branches (mm)	
Water	4.7	8.1	6.3	Not determined
YMB	4.1	7.7	6.4	0.48
Culture supernatant	6.6	10.0	6.3	0.56
Broth culture	6.7	10.6	6.2	0.61
LSD	0.93	1.65	0.6	0.044

out in experiments where strain 204 had a positive effect on the growth of barley indicated that the bacterium only rarely entered root tissue and if this did occur, the bacteria were found between and not within the cells. Thus strain 204 does not form the intimate relationship with barley that is required if its mechanism of action on the growth of cereals is through N₂ fixation (James 2000). Overall, the evidence indicated that strain 204 does not act on cereals by providing additional N via N₂ fixation.

There is considerable evidence that the primary mechanism of the stimulatory effect of *Azospirillum* and other PGPB on temperate cereal growth is the production of plant growth substances which cause changes in root morphology and physiology that result in increased nutrient and water uptake from the soil, especially during early growth (James 2000; Andrews et al. 2003; Mantelin and Touraine 2004). To assess if strain 204 acts on cereals via the production of plant growth substances, the effect of its culture supernatant on germination and growth of barley was examined. In experiment 1, which focused on seed germination and early growth, both strain 204 culture and culture supernatant gave greater shoot and root length in comparison with YMB or water (Table 2). Similarly, in Experiment 2 in the glasshouse, shoot dry weight was greater with strain 204 culture and culture supernatant than with YMB (Table 2). In Experiment 1, growth stimulation was similar with the application of culture or culture supernatant while in Experiment 2, the growth stimulation with the supernatant was around 70% of that with the culture (Table 2). These results indicate that the major proportion of the positive effect of strain 204 on growth of barley is due to

the production of plant growth substances. Greater growth of barley with strain 204 culture in comparison with culture supernatant may have been due to production of growth promoting substances by strain 204 in the pots.

Generally, the positive effects of PGPB on temperate cereal growth are associated with increased lateral root and root hair number and length. These effects have been linked with IAA production by the bacteria (Ona et al. 2003; Mantelin and Touraine 2004). In Experiment 1, neither bacterial culture nor bacterial culture supernatant affected root branching despite both causing increased root and shoot length (Table 2). Also, microscopy studies on seedlings and mature plants found no effect of strain 204 on lateral root or root hair production (data not shown) and IAA was undetectable (< 50 nM) in bacterial culture. Thus, production of IAA does not appear to be a factor in the mechanism of the growth effect of strain 204 on barley. Indeed, strain 204 may be restricted in its ability to produce IAA. For several bacterial species including *Azospirillum* spp., IAA is primarily synthesised from tryptophan (Ghosh and Basu 1997; Zakharova et al. 1999; El Khawas and Adachi 1999; Perrine et al. 2004) but in the phenotypic test carried out here, strain 204 did not produce indole from tryptophan.

Plant growth promoting bacteria have been reported to produce plant hormones other than IAA (Mantelin and Touraine 2004). In Experiment 1, both strain 204 culture and culture supernatant stimulated shoot and root growth (Table 1). This experiment was carried out in the dark and photosynthesis was not possible. Thus increased root and shoot extension indicates that a substance released by the bacterium resulted in

the increased rate of mobilisation of seed reserves. This is a common effect of GA (Briggs 1992). Analysis of strain 204 cultures gave an average concentration of $1.3 \pm 0.31 \mu\text{M}$ GA which is in the range previously reported to stimulate the rate of mobilisation of seed reserves of barley and several other plant species (Briggs 1992; Bajracharya 1999).

In conclusion, this study confirms the taxonomic assignment of strain 204 as a strain of *R. radiobacter*. However, in contrast to the findings of previous workers in Russia (Bairamov et al. 2001; Zavalin et al. 2001), we have been unable to demonstrate that the organism is diazotrophic. There was no evidence of acetylene reduction, moreover, no appropriately sized PCR products were obtained using nifH primers and no hybridisation of nifH probes to genomic DNA occurred. This is strong evidence that there is no nif gene present in strain 204 which is related to the nif genes present on the symbiotic plasmids of Rhizobial strains. The major proportion of the growth stimulation of barley obtained by application of strain 204 could be obtained by the application of its culture supernatant. It seems likely that the primary mechanism of the stimulatory effect of strain 204 on cereal growth is the production of plant growth promoting substances. In contrast with previous reports on PGPB, production of IAA appears not to be important in the mechanism of this growth effect. However, GA is likely to play an important role in stimulation of early growth of barley.

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