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(54) **Title:** NOVEL MICROORGANISMS AND THEIR USE IN AGRICULTURE

(57) **Abstract:** A strain of *Gluconacetobacter diazotrophicus* (Gd) characterised by the presence of at least one nucleic acid sequence selected from SEQ ID NOS 1-10 or variants or paralogues thereof and/or the presence of a single plasmid of about 17566 bp in size. Such strains, exemplified by IMI504853, are useful in agriculture, in particular as they are able to colonise plants intracellularly.



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Novel Microorganisms and Their Use in Agriculture

The present invention relates to novel microorganisms, specifically novel strains of the nitrogen-fixing bacteria
5 *Gluconacetobacter diazotrophicus* (Gd), and their use in agriculture, including agriculturally acceptable compositions containing these microorganisms. The strains have good utility in agriculture, in terms of their ability to colonise plant cells intracellularly, giving rise to particularly effective
10 nitrogen fixation. Kits for identification and monitoring of the use of these strains form the subject of a co-pending application.

Background of the Invention

Gluconacetobacter diazotrophicus (Gd) has been well
15 studied for its nitrogen fixing and plant growth promoting activities as reviewed in Eskin et al. International Journal of Agronomy (2014):1-13. Certain strains of Gd however have been shown to be particularly advantageous in the treatment of plants since they are able to establish themselves intracellularly
20 within plant cells along with exhibiting species and tissue independence (Cocking et al., In vitro Cellular & Developmental Biology Plant (2006) 42 (1). These properties, combined with their ability to travel throughout a range of plant tissues, make such strains better able to deliver the benefits to the
25 target crop plants.

However, a wide range of strains of Gd exist and it has not yet been possible to provide a means for easily identifying strains which have these beneficial properties.

Furthermore, an important aspect of bio-fertiliser has
30 been to provide an alternative to the chemical fertilisers in a nature friendly way to agricultural crop plants. However, it would be helpful to validate the effectiveness of any on-going treatment in field conditions, so that a farmer is able to determine what levels of nitrogen fertiliser, if any, are
35 required to be supplied to enhance growth conditions.

The applicants have found specific advantageous strains of Gd contain a number of unique nucleic acid sequences that are not found in other Gd species, nor in any other species, including plant species. This means that the beneficial strain can be readily identified and gives rise to the provision of a reliable diagnostic test, useful in both monitoring of treatments in the fields and in research, for identifying related beneficial strains.

Summary of the invention

According to the present invention, there is provided a strain of *Gluconacetobacter diazotrophicus* (Gd) characterised by the presence of at least one nucleic acid sequence selected from SEQ ID NOS 1-10 or variants or paralogues thereof and/or the presence of a single plasmid of about 17566 bp in size.

Strains having these unique characteristics have been found to be particularly effective in intracellular colonization of plant cells resulting in beneficial nitrogen fixing. In particular, it has been found that use of a strain of the invention leads to yield enhancements in crops such as cereal crops like maize and wheat. Alternatively, similar yields can be achieved even with reduction in traditional nitrogen fertiliser applications.

Sequences of SEQ ID NO 1-10 (shown in attached Table 1) hereinafter, are present in the particular strain IMI504853 but do not appear in genomic analyses of other available strains.

Variants of SEQ ID NOS 1-10 will include allelic forms which are highly naturally occurring variants. They will have a high level of sequence identity, for example, at least 70%, 70%, for instance at least 71%, 75%, 79%, 81%, 84%, 87%, 90%, 93%, 95%, 96% or 98% identical to the basic sequence. Identity in this context may be determined using the BLASTP computer program with SEQ ID NO 2 or a fragment, in particular a fragment as described below, as the base sequence. The BLAST software is publicly available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> (accessible on 27 July 2015).

In particular however the strain of the invention is characterized by the presence of at least one nucleic acid of SEQ ID NOS 1 -10, suitably at least 3 different nucleic acid sequences of SEQ ID NOS 1-10, for example at least 5 or at least 8 different nucleic acid sequences of SEQ ID NOS 1-10, and in particular by the presence of all nucleic acid sequences of SEQ ID NOS 1-10.

Preferred strains may also be characterised by the presence of a single plasmid of about 17566 bp in size. The presence of a plasmid, in particular of this size, differs from a previously known strain of Gd, UAP5541, which has been reported as lacking in plasmids (Luis E. Fuentes-Ramírez et al., FEMS Microbiology Ecology 29 (1999) 117-128). Furthermore, the size of the plasmid is smaller than that reported previously in respect of PAL5 strains containing a single plasmid (Giongo et al. Standards in Genomic Sciences, May 2010, Volume 2, Issue 3, pp 309-317 doi: 10.4056/sigs.972221).

The plasmid of strains of the invention may also be characterized in that it is restricted into two fragments by the restriction enzyme EcoRI, wherein the fragments are about 12 Kb and about 5.6 kb in size respectively. Furthermore, the plasmid lacks a number of key sequences which have been previously identified as being present in the plasmid of PAL5. These sequences are shown as SEQ ID Nos 65, 66, 67 and 68 in the attached sequence listing. Thus the absence of these particular sequences may provide a further characterizing feature of the strains of the invention.

A particular example of a strain of the invention is according to any one of the preceding claims which is IMI504853, deposited at CABI (UK) on 22nd May 2015.

Dot plot analysis of the plasmid of this strain was performed against the small plasmid (NC_010123; 16610 bp) from Pal5 DNA sequence analysis (M. Bertalan et al. BMC Genomics (2009) 10:450 DOI: 10.1186/1471-2164-10-450) using GEPARD software V 1.30 (Krusiek et al. Bioinformatics 2007; 23(8): 1026-8). Interestingly, no plot was produced when DNA sequences

of the two plasmids were compared. This indicates that the homology is so low that no structural similarity could be established by the software; thus confirming that the plasmid is a single and unique plasmid.

5 For use in agriculture, the strains of the invention are suitably formulated into an agricultural composition.

Thus a further aspect of the invention comprises an agricultural composition comprising a strain as described above in combination with an agriculturally acceptable carrier.

10 Generally the concentration of nitrogen-fixing bacteria that is present in the composition will vary depending upon factors such as the manner of administration, the type of plant or seed being treated and the particular strain of Gd used and the level of enhanced nitrogen-fixation required. Typically however, the
15 compositions will comprise a solution containing from 1 to 1×10^9 bacteria per millilitre of composition, for example from 10^1 - 10^3 bacteria per millilitre of composition for instance from 50-200 bacteria per millilitre of composition such as 100 bacteria per millilitre of composition. Such a composition may
20 be obtained by culturing the bacteria to a readily detectable level for example by examining the optical density and then diluting the solution accordingly.

The Gd may be the sole active component of the composition or it may be combined with additional agrochemically active
25 components such as insecticides, fungicides or plant growth regulators, provided these are compatible with Gd.

The composition of the invention suitably comprises a solvent such as water although organic solvents, such as vegetable oils or hydrocarbons such as paraffin or kerosene oils
30 may be used if required. Suitably any organic solvent is a vegetable oil such as soybean oil, sunflower oil, canola oil (oilseed rape oil), cottonseed oil, castor oil, linseed oil or palm oil or mixtures of these.

The composition may further comprise additives or
35 excipients such as thickening agents, dispersants, diluents, humectants, solid carriers etc. as are known in the art.

In a particular embodiment, the composition further comprises a polysaccharide or an agriculturally acceptable surfactant or a combination of these. The applicants have found that such components may enhance the activity of the
5 composition, as described in a co-pending British patent application. Suitable polysaccharides include hydrocolloid polysaccharides derived from plant, animal or microbial sources.

In particular, these include exudate gum polysaccharides such as gum Arabic, gum ghatti, gum karaya and gum tragacanth,
10 cellulosic derivatives such as carboxymethylcellulose, methylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose or microcrystalline cellulose, starches and derivatives including, for instance corn starch, tapioca starch, potato starch, rice starch, wheat starch, and modified versions
15 thereof such as pregelatinized starch, oxidized starch, ethylated starch, starch dextrans or maltodextrin, pectin, polysaccharides derived from seaweed such as agar, alginates, carrageenan, and fucellaran, seed gums such as guar gum and locust bean gum, polysaccharides derived from microbial
20 fermentation such as xanthan gum and gellan gum, and nitrogen containing polysaccharides such as chitosan; or mixture of these.

In a particular embodiment, the polysaccharide is exudate gum polysaccharide such as gum Arabic, gum ghatti, gum karaya or
25 gum tragacanth. A particular example of the polysaccharide is gum Arabic.

The amount of polysaccharide present in the composition may vary depending upon factors such as the manner of administration, the type of plant or seed being treated and the
30 particular strain of Gd used and the level of enhanced nitrogen-fixation required. This will vary depending upon the various factors such as the particular polysaccharide used, the type of plant or seed being treated, the particular strain of nitrogen-fixing bacteria employed and the method of administration.
35 However, typically, a composition comprising from 0.1 to 1%w/w,

for example from 0.1 to 0.5%w/w such as about 0.3%w/w polysaccharide is used.

In a particular embodiment, the composition further comprises a surfactant or detergent including in particular a non-ionic detergent such as those sold under the trade name 'Tween'®, for example Tween 80. Tween 80 is a non-ionic detergent; 70% composed of the fatty acid oleic acid and the remainder a combination of linoleic, palmitic and stearic acids. The pH of a 1% solution is in the range of from 5.5-7.2. It is widely used for emulsifying and dispersing substances in medicinal and food products. It has little or no activity as an anti-bacterial agent (Dawson et al. (1986) Data for Biochemical Research, 3rd ed., Oxford University Press (New York, NY: 1986), p. 289). The amount of surfactant included vary depending upon the various factors such as the particular surfactant used, the type of seed being treated, the manner of administration, the type of plant or seed being treated and the particular strain of Gd used and the level of enhanced nitrogen-fixation required. However, typically, a composition comprising from 0.0005 to 0.2%v/v for example from 0.0005 to 0.15%v/v such as about 0.001%v/v.

Suitably a nutrient for the Gd is also included in the composition. Examples include a 3%w/v sucrose solution as described in EP-B-1422997.

Since by and large, all the components of the compositions are natural products, the environmental impact of the treatments of the invention is low and the compositions may satisfy regulatory requirements relatively easily. In a particular embodiment, the formulation is applied as a seed coating as described above. In particular, the formulation is applied to seeds, either by spraying or by soaking and the seeds are then dried to form a residual coating comprising Gd thereon.

A further aspect of the invention provides a method for enhancing the nitrogen-fixing ability of a plant, said method comprising administering to the plant or to the environment thereof, a strain of Gd as described above, or an agricultural

composition comprising it so that the Gd colonises plants and in particular enters plant cells (intracellular colonisation).

This may be achieved in a variety of ways. For example, the Gd may be administered to a growth medium of the plant, in particular on or after germination, using a method as described for example in EP-A-1714545. In this case, administration of in particular low levels for example from 1-100 bacteria per millilitre of inoculum to the growth media such as agar is applied to grasses, in for example on germination or shortly thereafter, for example up to 7 days thereafter.

Alternatively, the Gd may be applied to a seed in particular to the surface of a seed, for example in a pre-germination soak or, in a particular embodiment, as a seed coating. Alternatively, the one or more compositions may be applied to a growth medium on which the seeds are allowed to germinate. Such growth media include artificial media such as agar gels as well as soils or composts in which the seeds are about to be or have been sown.

In yet another embodiment, the Gd is applied to growing plants in and particular to a wound of the plant. This technique is described in the applicants copending International Patent Application No PCT/GB2015/052170. In this method, the nitrogen-fixing bacteria is administered to a wound of a growing plant. The wound may be a result of accidental or natural damage, whereupon the additional nitrogen availability may facilitate repair growth. However, in a particular embodiment, the wound is the result of damage caused by actions such as mowing (amenity grass), cutting (silage and hay crops), consumption by livestock (pasture grass) or by harvesting. Therefore, a preliminary step of inflicting 'damage' on the grass, in particular by mowing, cutting, or by harvesting may be carried out before administration of the nitrogen-fixing bacteria. The nitrogen-fixing bacteria is suitably applied within a relatively short time period of carrying out such actions, for instance, within 48 hours, for instance within 24

hours, such as within 10 hours and suitably within 1-2 hours of damage being inflicted on the plant.

Delivery of the bacteria is achieved by application of a suitable formulation to the wound area in particular in the form of a composition. The composition may be in the form of a liquid, gel, paste which may be applied directly or in diluted form, or it may be in the form of a solid composition such as a powder or granule composition that will be dissolved in liquid such as water before use. In solid compositions, the bacteria will generally be used in dried form, for example in freeze-dried form, which are reconstitutable on addition of water. However, the desiccation resistance of the present strains may mean that freeze-drying is not required.

In a particular embodiment, the composition is in a form suitable for spraying on the plants and thus will comprise a concentrate for dilution which may be in the form of a liquid or solid, in particular in the form of a liquid, or it may comprise a dilute aqueous composition that may be sprayed directly.

The amount of nitrogen-fixing bacteria that is administered in any particular case will vary depending upon factors such as the type of seed or plant being treated, the particular strain of nitrogen-fixing bacteria used, the level of germination enhancement required and the method of administration. Typically however, the seeds or the environment thereof such as the growth medium is inoculated with a solution containing from 1 to 1×10^9 bacteria per millilitre of inoculum, for example from 1-100 bacteria per millilitre of inoculum, for instance from 10-80 bacteria per millilitre of inoculum such as 50 bacteria per millilitre of inoculum. Such a solution may be obtained by culturing the bacteria to a readily detectable level for example by examining the optical density and then diluting the solution accordingly.

In a particular embodiment, the Gd is administered together or in combination with a strain of *Terribacillus*, as described in the applicants co-pending International patent application No. PCT/GB2015/052171. The applicants have found that

such a strain may enhance the activity of the Gd. Suitable strains of *Terribacillus* include *Terribacillus saccharophilus*, *Terribacillus halophilus*, *Terribacillus goriensis* or *Terribacillus aidingensis* but in particular is a strain of *Terribacillus saccharophilus*. The *Terribacillus* is administered either separately or in admixture with the Gd. The *Terribacillus* may be in intimate admixture with the Gd, or it may be administered in a co-culture, or mixed culture form.

Suitable plants include leguminous and non-leguminous crops and ornamentals. The non-leguminous plant is preferably selected from the grass family Gramineae (includes rice [*Oryza sativa*], wheat [*Triticum aestivum*] and maize [*Zea mays*]). The non-leguminous plant may also be one selected from families such as: Solanaceae (includes tomato, potato and tobacco), Brassicaceae/Cruciferae (includes cabbages, turnips, oilseed rape and the model plant *Arabidopsis thaliana*), Malvaceae (includes cotton), Compositae/Asteraceae (includes sunflower and lettuce), Euphorbiaceae (includes cassava), Chenopodiaceae (includes sugar beet). The leguminous plant is preferably selected from the Leguminosae (includes soybean, clover, alfalfa, peas and other beans). Seeds treated may be of monocotyledonous or dicotyledonous plants but in particular are monocotyledonous plants such as grasses, rice, wheat, barley, sorghum, millet, oats, rye and maize. In particular the seeds treated in accordance with the method of the invention are grasses such as *Lolium perenne*.

Other crops listed above may use the Gd to facilitate survival in non-optimal pH conditions as described above.

Once inoculated as described above, the Gd of the invention will colonise the plant cells and act symbiotically within the plant to enhance nitrogen-fixation.

Thus a further aspect of the invention provides a plant or seed which has been colonised by a strain of Gd as described above. In particular the Gd is located intracellularly in living plant cells.

The applicants have found that intracellular Gd will pass from generation to generation and that thus seeds and other progeny obtained from the seeds and plants of the invention will also comprise intracellular Gd. Such seed or progeny form yet a further aspect of the invention.

By enhancing nitrogen-fixation, plants may show enhanced properties, such as more rapid or improved growth characteristics and/or improved yield. In a further aspect, the invention provides a method for producing a plant product, said method comprising growing a plant colonised with Gd as described above, and obtaining the product therefrom.

In addition, the applicants have noted that certain plants colonised by nitrogen-fixing bacteria, in particular intracellularly colonised plants, show not only enhanced growth or yield characteristics, but also show an increased chlorophyll level as compared to plants without such intracellular bacteria. The plants which appear particularly to demonstrate this property are grasses including pasture, amenity or turf grasses. This is particularly advantageous in the context of such plants as high chlorophyll levels give rise to a better green colouration, which is highly desirable in an ornamental context.

Thus, in a further aspect, the invention provides a method for increasing chlorophyll in grasses, which method comprises growing grasses which comprise Gd as described above.

The grasses may include grasses such as pasture, amenity, turf or ornamental grasses, for example, *Lolium* spp, such as *Lolium perenne*, *Lolium multiflorum*, *Lolium persicium*, *Agrostis* spp. such as *Agrostis castellana*, *Agrostis capillaris*, *Agrostis stolonifera*, *Festuca* spp such as *Festuca rubra*, *Festuca ovina*, *Festuca longifolia*, *Festuca arundinacea*, *Poa* spp such as *Poa annua*, *Poa Pratensis* and *Poa trivialis*, *Paspalum vaginatum*, *Cynodon* spp such as *Cynodon dactylon*, *Zoysia* spp such as *Zoysia japonica*, *Zoysia tenuifolia*, or Emerald *Zoysia*, *Stenotaphrum secundatum*, *Buchloe dactyloides* or *Pennisetum clandestinum*. Generally however, *Lolium* spp. and/or *Festuca* spp. form the basis of many amenity or turf grasses.

The Gd may be administered to the grasses using any of the techniques described above, but in particular, may be applied to the seeds of the grasses as a coating, or to wounds of the grass, inflicted after cutting.

5 Strains in accordance with the invention may be identified and/or monitored by use of a diagnostic kit comprising means to determine the presence in a sample of at least one nucleic acid sequence selected from SEQ ID NOS 1-10.

10 As discussed above, SEQ ID NOS 1-10 represent unique and novel sequences, which appear in a preferred sub-species or strain of Gd. Thus they provide a means for identifying these beneficial strains. In addition, they have been found to be amenable to detection using primers which do not cross-react with plant species.

15 Furthermore, since the strains can colonise intracellularly in the host plant cells, they are able to effectively travel throughout the plant. The kit of the invention can be used to provide an evaluation of colonisation by Gd post-germination at a young stage and check its
20 efficiency. This will then allow the farmers to make an "informed decision" about the existence and extent of the colonisation and if required how much chemical based nitrogen fertiliser will be required to be applied. This extra level of security will provide 'assurance' to farmers that their crops
25 are being well-tended, even when using nature and crop friendly fertiliser in the form of Gd.

 In a particular embodiment, the kit may comprise means for determining the presence of more than one of the nucleic acid sequences of SEQ ID NOS 1-10, for example up to 10, such as up
30 to 5, or up to 3 of said nucleic acid sequences of SEQ ID NOS 1-10. In this way, a reliable diagnostic kit would be provided which will ensure that the strain detected is the one which is similar or substantially similar to the beneficial strain. Similar strains would be detected even if one or more of the
35 sequences differs for example as result of mutation.

Preferred strains appear to contain a plasmid, and so plasmid detection, for example by isolation using a commercially-available plasmid isolation kit, may provide further confirmation of the identification of a beneficial strain. In particular, any plasmid identified should be less than 27455 bp in size, for example about 17566 bp. The presence of a plasmid, in particular of this size, differs from a previously known strain of Gd, UAP5541, which has been reported as lacking in plasmids (Luis E. Fuentes-Ramírez et al., FEMS Microbiology Ecology 29 (1999) 117-128). Furthermore, the size of the plasmid is smaller than that reported previously in respect of PAL5 strains containing a single plasmid.

The plasmid may be subject to cutting, in particular by means of an EcoRI restriction enzyme, to yield two fragments of about 12 kb and about 5.6 kb.

The diagnostic kit may further comprise means for determining the presence of at least one nucleic acid sequence which is characteristic of Gd species, for example 2, or 3 nucleic acid sequences which are characteristic of Gd species. In this way, the kit would provide confirmation that some Gd is present in the sample, thus confirming the accuracy of the test. If the sample is known to contain Gd, a positive result in this determination would act as a 'control' confirming that the test has been carried out effectively. Suitable specific strains will be sequences found in Gd species generally but not in other species, and in particular not in other microbial species or in at least some plants, in particular plants which may be targeted for Gd treatments.

Particular examples of such sequences are shown as SEQ ID NOS 11-13 in Table 2 hereinafter. In particular, these nucleic acid sequence which is used to detect Gd species have been found to be amendable to detection of Gd species present in a range of crops, without cross-reacting with plant species.

In addition, the kit may comprise means for detecting sequences which are not found in the strain of the invention, to provide a negative control. Examples of such sequences are shown as SEQ ID Nos 65-68. In a further embodiment, the kit comprises means for

detecting the presence of a plant specific nucleic acid sequence, such as a chloroplast specific nucleic acid which amplifies universally from plant DNA. The inclusion of such means will act as a control when the kit is used in the context of detection of Gd within a plant species, as this means will produce a detectable signal, even in the absence of any Gd. If this signal fails, this would indicate failure in the test rather than necessarily that there is no Gd present. A particular chloroplast primer set which is available commercially from Thermo Scientific. Product as Phire Plant Direct PCR Master Mix, is based on the disclosure by Demesure B et al (1995) Molecular Ecology 4:129-131.

The means for detecting the presence of the nucleic acid sequences may take various forms as would be understood in the art.

Where the nucleic acids are genes which are expressed, the kit may comprise means for detecting the expressed proteins. Such means may include specific protein tests such as immunochemical analysis which utilise antibodies specific to the proteins to immobilise and/or detect the proteins, such as ELISA or RIA techniques, or immunoelectrophoretic methods such as Western blotting.

However, in a particular preferred embodiment, the kit of the invention comprises means for detecting specific nucleic acids themselves.

In particular, nucleic acids may be detected using any of the available techniques including nucleic acid binding assays and immunoassays using antibodies raised to haptenised forms of the nucleic acids. However, in a particular embodiment, the detection involves nucleic acid amplification reactions, and thus in particular, the kits will comprise one or more amplification primers which target the or each nucleic acid sequence being detected.

As would be understood by a skilled person, when detection of a sequence is carried out using an amplification reaction, it would not be necessary to amplify the entire sequence, but

rather just a characteristic fragment of the entire sequence, for example a fragment of at least 10, and suitably at least 50 base pairs. Thus the size of the sequence that may be amplified would could be in the range of from 10-3070 base pairs, and
5 suitably from 20-2000 base pairs, for example from 50-500 base pairs, such as from 100-300 base pairs. The size of the fragment will depend upon the nature of the detection reaction being used, but it should be sufficient to ensure that the product is characteristic of SEQ ID Nos 1-10 or variants as
10 defined above, and so is not present in other sequences, in particular plant sequences. Where more than one sequence is amplified, they may be selected to be of differing sizes, so that they may be easily differentiated during detection, using techniques such as separation on the basis of size, or melting
15 point analysis.

If required, the kit may further comprise one or more additional reagents necessary to carry out a nucleic acid amplification reaction. Thus it may include enzymes such as polymerases, salts such as magnesium or manganese salts, buffers
20 and nucleotides as would be understood in the art.

The kits may, if required, include means for detecting the products of the amplification. Such means may include dyes or probes, in particular labelled probes that bind the target sequence intermediate the primers. Alternatively, the primers
25 themselves may be labelled to facilitate detection.

Suitable nucleic amplification reactions include reactions that utilise thermal cycling such as the polymerase chain reaction (PCR) and ligase chain reaction (LCR) as well as isothermal amplification reactions such as nucleic acid sequence
30 based amplification (NASBA), strand displacement amplification (SDA), transcription mediated amplification (TMA), loop-mediated isothermal amplification (LAMP) and rolling circle amplification, 3SR, ramification amplification (as described by Zhang et al., Molecular Diagnosis (2001) 6 No 2, p 141-150),
35 recombinase polymerase amplification (available from TwistDx) and others. In a particular embodiment, the nucleic acid

amplification is a PCR, and may be a quantitative PCR (QPCR) to provide information regarding the extent of colonisation.

In an alternative embodiment, the amplification is LAMP reaction. LAMP assays utilise at least four and suitably six
5 primers which are designed to target six different regions of the target sequence. There will always be two outer primers (F3 and B3) and two inner primers (FIP and BIP). Optionally, in addition there are two loop primers (FLoop and BLoop). The use of the Loop primers usually reduces the amplification time and
10 increases the specificity of the assay. FIP and BIP primers consist of F2, complementary to the F2c region of the template sequence, and F1c, identical to the F1 region of the template. Four main features need to be considered in order to guarantee a successful LAMP primer design: the melting temperature of the
15 primers (T_m), given 55–65°C for F3, FIP, BIP and B3 primers and $\geq 65^\circ\text{C}$ for FLoop and BLoop; a GC content of 50–60% in the primer sequences; the absence of secondary structures formation and stability at the ends of each primers; and finally the distance between primer regions. Examples of suitable LAMP primer sets
20 are disclosed hereinafter.

The presence of the products of amplification reactions may be determined using any available technology. Thus they may include techniques where products are separated on a gel on the basis of size and/or charge and detected such as agarose gel
25 electrophoresis. Alternatively, they may be detected in situ, using for example using intercalating dyes or labelled probes or primers. The detection of amplification products using a wide variety of signalling and detection systems is known. Many of these systems can be operated in 'real-time', allowing the
30 progress of amplification to be monitored as it progresses, allowing for quantification of the product. Many such systems utilise labels and in particular fluorescent labels that are associated with elements such as primers and probes used in the amplification system and which rely on fluorescent energy
35 transfer (FET) as a basis for signalling. A particular form of such fluorescent energy transfer is fluorescent resonance energy

transfer or Förster resonance energy transfer (FRET) for signal generation.

A major example of such a process used commercially is the TaqMan® process, in which a dual-labelled probe, carrying both a
5 first label comprising a fluorescent energy donor molecule or reporter and a second label comprising a fluorescent energy acceptor molecule or quencher, is included in a PCR system. When bound to the probe, these molecules interact so that the fluorescent signal from the donor molecule is quenched by the
10 acceptor. During an amplification reaction however, the probe binds to the target sequence and is digested as the polymerase extends primers used in the PCR. Digestion of the probe leads to separation of the donor and acceptor molecules, so that they no longer interact. In this way, the quenching effect of the
15 acceptor is eliminated, thus modifying emissions from the molecule. This change in emission can be monitored and related to the progress of the amplification reaction.

Where more than one nucleic acid sequence is detected, the kit may comprise components sufficient to carry out multiple
20 separate amplification reactions, such as individual sets of primers. Preferably however the kit is set up to carry out a multiplex reaction, where multiple targets may be detected in a single reaction. In this case, where the detection is done using gel electrophoresis, the primers are suitably selected so that
25 each amplified product has a significantly different size or charge so that they may be readily separated and identified on an agarose gel, or by melting point analysis using a signalling reagent such as a DNA intercalating dye.

Alternatively, where the detection system includes labels,
30 any labels provided for example on primers or probes, will provide a different and distinguishable signal from other primer sets, for example on the basis of the wavelength of the emitted signal and/or the fact that the product has a different melting point or annealing temperature, which may be
35 distinguished by carrying out a melting point analysis of products.

Suitable amplification primers, in particular for PCR amplification, together with the approximate size of the products they generate are selected from those set out in Table 3 below:

5

Table 3

	SEQ ID	Forward	SEQ ID	Reverse	Product size
A	14	TGAAATTGACGCCCGTTGGA	15	CACGCCGGGAAAGAGGATTC	472 bp
B	16	GGCAACGCGGTTTCTACGAA	17	CGTTAGCCGGGGTTGTCAGA	489 bp
C	18	TCGTTGCCACTTTCCGAGGG	19	GTCGATTGTGTGCAGCGTCA A	268 bp
D	20	CACCGATCTTGTGCGTTTCG	21	CGGCAATGCTCCATACCCAC	522 bp
E	22	CACCGGAAAGAGTGGCAGGA	23	AACCGGGTCACTTGCCTCAT	783 bp
F	24	AGCCATCGGAGTCACATCGG	25	GGAAACCTCGAAACCCTGCG	1129 bp
G	26	TCAGGGCAATCACTAGCCGG	27	TCGAGCAGCCGTTTCATCCA	1118 bp
H	28	TGATGCGCTTGTTGCTGACG	29	CGTTCGCCCTTGTCGTCATG	478 bp
I	30	GGGCCATCCGTTACCTGCTT	31	TGACACACCCGCTCCGAAAT	1102 bp
J	32	GCATTTGCGGTAAGTCATCC CA	33	GGATCCCGATTTGCAAGCCA	814 bp
K	34	TGTCGGGTCGGGAAGTCAAG	35	CGGGTTCTCGCTGATGACCT	464 bp
L	36	TCCCGCCTGCATCTGAAGAC	37	CAGCGATGCCAGCCAATACC	1098 bp
M	38	GTTCGTCGCGTCTGATGCAG	39	ACCTGGGCATTGTTGGTGGA	1045 bp

Primer sets represented by SEQ ID NOS 14-33 have been found to act as useful strain-specific primers for beneficial strains of *Gd*, while primer sets represented by SEQ ID NOS 34-39 act as useful *Gd* species-specific primers.

The kits may be used in methods for determining the presence in a sample of a strain of *Gluconacetobacter diazotrophicus* (*Gd*) able to intracellularly colonise plant cells, said method comprising detecting in said sample at least one nucleic acid sequence selected from SEQ ID NOS 1-10.

Suitably up to 10, for example up to 5 such as about 3 of said nucleic acid sequences of SEQ ID NOS 1-10 are detected.

The method may further comprise detecting at least one nucleic acid which is characteristic of Gd species, as described above, such as a nucleic acid sequence of SEQ ID NOS 11-13. Again, more than one such species specific nucleic acid sequence
5 may be detected if required.

In addition, the method may further comprise detecting a plant specific nucleic acid sequence, which may be characteristic of the particular Gd colonised plant being examined or may be universally present in plants, such as a
10 chloroplast specific nucleic acid sequence, as a control for the reaction.

Various methods of detection may be used in the method, as described above, but in particular, the method comprises a nucleic acid amplification reaction, such as the polymerase
15 chain reaction (PCR).

Suitable primers are as described above.

The sample which may be used in the method of the invention may be any sample that contains or is suspected of containing a strain of Gd. This may include cultures or
20 laboratory samples, which may contain the desired strain of Gd. Alternatively, they may comprise plant samples, including leaf, stem, or root samples, from which nucleic acid has been released, for example by causing cell lysis, for example using mechanical, chemical or sonic means. In particular, the sample
25 is from a plant to which a strain of *Gluconacetobacter diazotrophicus* (Gd) has previously been applied. In this way, the successful colonisation of the plant by Gd can be confirmed.

Typically, such tests will be carried out in a laboratory, although mobile testing, for example, in field conditions, may
30 be carried out if suitable equipment, such as mobile PCR machines, are available, or if detection of targets in particular protein targets, using techniques such as ELISAs, which may be carried out on lateral flow devices are employed.

Detailed Description of the Invention

The invention will now be particularly described by way of example with reference to the accompanying figures which are described as follows:

- 5 **Figure 1:** is a gel showing PCR products from a range of designed to be strain or species specific, including primer sets A-H in Table 3 (shown in Figure 1A in lanes 4, 5, 6, 7, 8, 9, 12 and 13 respectively) and I-M in Table 3 shown in Figure 1B in lanes 4, 5, 9, 12 and 13 respectively.
- 10 **Figure 2:** is a gel illustrating PCR products using Primer set E following inoculation of reactions with 100 ng of (1) OSR var. Ability, (2) OSR var. Extrovert, (3) rice var. Valencia, (4) wheat var. Willow, (5) grass var. Aberglyn, (6) grass var. Dickens, (7) maize, (8) quinoa, (9) Arabidopsis var. Columbia, 15 (10) barley var. Chapeaux, (11) grass var. Twystar, (12) grass var. J Premier Wicket, (13) potato, and (14) tomato. Lane (15) contains amplicon produced from 10 ng genomic DNA from Gd, (16) contains the no template PCR control, and the molecular weight marker at each end of the gel is Hyperladder 1 kb plus 20 (Bioline).
- Figure 3:** is a gel illustrating the sensitivity PCR using Primer set B in reactions containing 100 ng DNA from OSR var. Ability, co-inoculated with (1) 1 ng, (2) 100 picogram, (3) 10 picogram, (4) 1 picogram, (5) 100 femtogram, (6) 10 femtogram, and (7) no 25 added genomic DNA from Gd. Lane (8) is the no template control sample and molecular weight marker at each end of the gel is Hyperladder 1 kb plus (Bioline).
- Figure 4** are graphs showing (A) positive amplification of *Gluconacetobacter diazotrophicus* by fluorescent LAMP using the 30 Genie II real-time machine and a primer set embodying the invention. Positive DNA amplification is detected by a fluorescence signal, and (B) an anneal curve for the *Gluconacetobacter diazotrophicus* samples, following amplification by LAMP; the reaction was put through an anneal 35 analysis and the temperature at which the dsDNA reanneals is detected as a burst of fluorescence.

Figure 5 are graphs showing representative results of QPCR experiments carried out using primers designed to amplify sequences according to the invention, when carried out using serial dilutions of samples containing GD DNA, where (A) shows the results for primer set designated P5 for SEQ ID NOs 58 and 59, (B) shows results for a primer set designated P8 for SEQ ID NOs 60 and 61 and (C) shows results for a primer set designated P17 for SEQ ID NO 62 and 63 as defined hereinafter.

Figure 6 are graphs showing representative results of QPCR experiments carried out using primers designed to amplify sequences according to the invention, when carried out using serial dilutions of samples containing GD DNA and plant genomic DNA, where (A) shows the results for primer set designated P5 in the presence of wheat DNA, (B) shows melt peak graphs of the products of (A) for all the samples (i.e. dilutions of Gd in presence of wheat genome and relevant controls); (C) shows melt peak graph of the controls from (A) where a positive control comprising Gd DNA only resulted in giving signal, and negative controls comprising plant DNA only and QPCR negative samples only (NTC - no transcript control), both did not result in giving signal; (D) shows results for a primer set designated P17 as defined hereinafter in the presence of maize DNA; (E) shows the melt peak graph of the products of (D) for all the samples tested (i.e. dilutions of Gd in presence of Maize genome and relevant controls); and (F) shows the melt peak graphs of the controls from (D) where a positive control comprising Gd DNA only resulted in giving signal, and negative controls comprising plant DNA only and QPCR negative samples only (NTC - no transcript control), both did not result in giving signal.

Figure 7 shows a resolved 1% agarose gel showing plasmid DNA extracted from a particular strain of GD (IMI504853).

Figure 8 shows resolved 1% agarose gel restriction digestion product of plasmid DNA with EcoRI from strain of Gd (IMI504853). The restricted fragments are mentioned as 1) ~12Kb and 2) ~5.6Kb when run alongside 1kb Hyper ladder (Bioline) where the nearest fragment from the ladder is highlighted for the size comparison.

Figure 9 shows an agarose gel obtained from a PCR amplification to detect Gd from the seedlings of wheat obtained during a field trial.

Figure 10 is a table showing the chlorophyll index of wheat
5 treated with Gd in accordance with the invention compared to a control.

However, it will be apparent to one skilled in the art that the specific details are not required in order to practice the invention. The following descriptions of specific
10 embodiments of the present invention are presented for purposes of illustration and description. They are not intended to be exhaustive of or to limit the invention to the precise forms disclosed. Many modifications and variations are possible in view of the above teachings. The embodiments are shown and
15 described in order to best explain the principles of the invention and its practical applications, to thereby enable others skilled in the art to best utilize the invention and various embodiments with various modifications as are suited to the particular use contemplated.

20 Example 1

Identification of Unique Sequences

IMI504853, a Gd strain derived from passaging UAP5541, which was found to have particularly beneficial plant colonisation properties was isolated and the full genome
25 sequenced. A comparison was made against the publically available genome of the type strain (PAL5; sequenced by JGI, USA [Genbank sequence accession CP001189]) using standard methods.

Surprisingly, a large number of differences were noted in the genome, and in particular, a number of genes were identified
30 which are present in the genome of IMI504853 but not PAL5.

Many of these genes were annotated with an associated function. The unique genes with annotations were further checked for uniqueness across all the genomes sequenced to date using the NCBI's web-based BLAST tool
35 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Analysis of the BLAST result narrowed the list to 20 unique genes not present in any genome. These unique genes appeared to be "strain-specific" for IMI504853.

Also, five sets were found to be unique to the *Gd* species and will hereafter be referred to as "species-specific" (i.e. present in IMI 504853, Pa15 and other *Gd* strains but in no other species).

Thus these sequence differences appear to characterise the strain and can be used to design a diagnostic kit for IMI504853 and similar strains.

Example 2

PCR validation

A set of 25 primer sets were designed based upon the sequences identified in the analysis of the genome. The specificity of these 25 primer sets (20 designed to be strain-specific and 5 designed to be species-specific) were first tested by carrying out a conventional PCR reaction using genomic DNA of IMI504853 and PAL5. The results with IMI504853 are illustrated in Figure 1. Results showed that 16 strain-specific primer sets delineated IMI504853 from PAL5 as obtained from three different collections (ATCC49037, DSM5601 and LMG7603). However, 4 putative strain-specific primer sets cross-reacted with at least one PAL5 and hence were removed from strain-specific study.

All 5 species-specific primers reacted as expected.

Further, testing of strain- and species-specific primers was done against two other strains of *Gd*, one originally isolated in India (IMI 502398) and the other from Mauritius (IMI 502399), as well as a revived 2001 culture of UAP5541 strain (stored in glycerol at -80°C), using the method described above. The data was in agreement with the 16 strain specific primers and 4 species specific primer sets as one of the species-specific primer sets produced a higher molecular weight band. This was a surprise result.

The sensitivity of detection of all 25 primer sets (20 strain-specific and 5 species-specific) was checked using serial

dilutions of bacterial broth cultures. It was found that 24 of these sets produced very high levels of detection (requiring 1-10 bacterial cells).

Further, these 25 primers were then checked for cross-reactivity with several target plant species and varieties using DNA extracted in-house. The primers were tested in a PCR reaction using DNA extracted from plants of the following species: maize, wheat (var. Willow), quinoa, rice (var. Valencia), barley (var. Chapeaux), potato, Arabidopsis (var. Columbia), oilseed rape (vars. Ability and Extrovert) and a range of grasses (vars. Aberglyn, Dickens, J Premier Wicket, and Twystar). The method for isolating nucleic acids from plant tissues involved the mechanical maceration of leaf material followed by a modified CTAB extraction (Doyle and Doyle, 1987 Phytochem. Bull., 19: 11-15). Briefly, cellular membranes were disrupted using SDS and CTAB to release their contents, and cellular proteins were degraded or denatured using proteinase K and β -mercaptoethanol. The extraction buffer also contained PVPP to remove plant polyphenols, EDTA to chelate metal ions, sodium chloride to solubilise nucleic acid structures, as well as TRIS HCl to stabilise the buffer pH. RNA molecules were degraded using RNase A treatment. Following the removal of insoluble cellular debris using chloroform:isoamyl mix (24:1), deoxynucleic acids were precipitated in ethanol using sodium acetate, washed using diluted ethanol, and resuspended in molecular grade water.

Illustrative results are shown in Figure 2.

At the same time, the sensitivity of primers were tested by co-inoculating PCR reactions containing 100 ng of the above mentioned plant genomes with six-fold serially diluted genomic DNA from Gd, starting from 1 ng. It was found that the sensitivity of the PCR system was generally unaffected by the presence of plant genome and routine detection was established from a minimum of 1 picogram of Gd DNA.

Illustrative results are shown in Figure 3. Results suggested that 17 of the 20 strain-specific primer sets and

three of the five species-specific sets either do not cross react with any plant genomes tested, or cross-react with a small number but produce a DNA product of a different size and distinguishable size.

- 5 Of the strain-specific primer sets, only 10 produced results which were of (1) high specificity, (2) high sensitivity, and (3) produced no cross-reactions with plant DNA and these are represented in Table 3 above as primer sets A-J. Similarly only three of the selected species-specific primer
- 10 sets were found to be specific and sensitive enough for use and these are shown as primer sets K-M in Table 3 respectively. In addition, the size of the products obtained using these primers is shown in Table 3 and illustrated in Figures 1A and 1B. Thus, methods and kits based upon these primers are particularly
- 15 useful in identifying beneficial Gd in field situations.

Example 3

LAMP Assay

- A series of LAMP primers were designed to amplify regions of SEQ ID NOS 6, 7 and 9 and are shown in Table 4 below as
- 20 follows:

Table 4

SEQ ID NO	Sequence	Type
40	CTCAGGAAGACCGAATTGATTA	F3
41	GCGAAACGTCTGATTGAAC	B3
42	CGGATAACCACTGGTGCTCCGACTCGCCTCACTCTACT	FIP
43	TCCACGAATCTCACGAAGCACCCCGACCTTATCTCCCAT	BIP
44	GCCAGGCGTGTACATATAACTA	FL
45	CGGAATACCTAGTTGGAACACT	BL
46	TCAAGATCGATGCACCTATTC	F3
47	AACAGACAGTTCTGGTAGGA	B3
48	CGCATCTCCAGATCGGCAGGTCGTCCAGTCGATCATG	FIP
49	ACATCTGTCCACGGCATTGGTGGCTGGCTTATGAGTCT	BIP
50	GAGAAGTCCTCTGCTTCGG	FL
51	CGGCGGTTGAGAAGATGT	BL

SEQ ID NO	Sequence	Type
52	GGAAGACATCAACGAAGCA	F3
53	TTGACAGTTGCATAGTCCG	B3
54	ATACGGCTCGTCATGTCGCGGTGATGGATAATCTCAGCC	FIP
55	CAGTGGCCGAACCTGGAAGCGCTGATATAAGCCTGAAGAT	BIP
56	ATTGCACCGCGTTGATG	FL
57	GCGTAACGGTCACAAGGA	BL

SEQ ID NOS 40-45 were designed to amplify SEQ ID NO 6 above, SEQ ID NOS 46-51 were designed to amplify SEQ ID NO 7 above, and SEQ ID NOS 52-57 were designed to amplify SEQ ID NO 9 above.

These primers were obtained and tested in a LAMP assay on samples comprising pure Gd DNA that had been isolated using a modified CTAB methodology from bacteria grown in liquid culture.

In addition, DNAs from a range of plant pathogenic bacteria and fungi was tested for amplification in LAMP by the primer sets. These included *Bacillus subtilis*, *Lactobacillus*, *Fructobacillus*, *Pseudomonas* spp., *Agrobacterium* spp., a range of phytoplasmas and various fungi including species from the *Fusarium*, *Penicillium* and *Aspergillus* genera.

Real-time LAMP was carried out on a Genie II instrument (OptiGene), and 1 µl of sample was added to a 24 µl reaction mix containing 15 µl Isothermal Master Mix ISO-001 (OptiGene), 200 nM of each external primer (F3 and B3), 2 µM of each internal primer (FIP and BIP) and 1 µM of loops primer (FLoop and BLoop). RT-LAMP reaction consisted of 30 minutes of isothermal amplification at 63°C. To evaluate the annealing temperature of the products, reactions were then subjected to a slow annealing step from 95 to 68°C (0.05°C/s) with fluorescence monitoring.

Negative reaction controls, consisting of water, were also used.

Of the three sets of primers tested in LAMP, the third primer set specific for SEQ ID NO 9 gave amplification in nine and a half minutes with an anneal at 89.2°C (see Figure 4A). The primer set specific for SEQ ID NO 6 amplified the positive

control at around 11 minutes with an anneal of approximately 88°C, and the primer set specific for SEQ ID NO 7 was the slowest, amplifying the positive control at around 23 minutes with an annealing temperature around 90°C.

- 5 All sets of primers that gave the positive Gd amplification were specific for the bacterium and did not amplify from DNA of any of the other bacterial and fungal DNAs they were tested on. They are therefore all suitable as primer sets to be used for detection of the Gd bacterium.

10 Example 4

Detecting Gd on plant samples using LAMP

- To validate the primers on rapidly extracted DNA from contaminated seed, a series of experiments were set up in which seed of two plant species, tomato and wheat, were spiked on the surface with Gd DNA. The samples were then put through the 2-minute DNA extraction technique in which the samples are placed in plastic tubes containing steel beads and TE buffer and shaken vigorously for 2 minutes. Two microliters of the solution was then placed in the LAMP reaction as described in Example 4 using the primer set comprising SEQ ID NOS 52 to 57 to test for amplification of the Gd DNA from these samples.

The results showed that the Gd DNA is detectable when put through these assays, against a background of plant DNA.

- In order confirm that any samples that tested negative for Gd supported LAMP amplification (*i.e.* they do not contain inhibitors of LAMP reactions), the cytochrome-oxidase gene (COX) primers (Tomlinson *et al.*, 2012 *Journal of Virological Methods*, **191**: 148-154.), which amplify DNA from the host plant, were used as controls for false negatives on all samples.

30 Example 5

QPCR Determination

- A range of QPCR reactions were carried out on samples comprising known quantities of DNA from Gd(IMI504853) and also from a range of crop species including maize, barley and wheat genomic DNA.

QPCR reaction mixtures were prepared to a volume of 20 μ L volume per reaction. In the case of Gd DNA alone, these consisted of 10 μ L iTaq™ Universal SYBR Green® Supermix (2X) (Bio-Rad), 1 μ L each of forward and reverse primers (final
 5 concentration of 10 μ mol), 7 μ L SDW (sterile distilled water) and 1 μ L DNA template at the required concentration.

Primers used in this case were as set out in Table 5.

Table 5

SEQ ID NO	Sequence	Type
58	AGGAGGCTCTTTCTTGAAGC	Forward
59	AAGTGCCCTGTTATCGTACAC	Reverse
60	TGGGTCATCGGTTCTGATTTCC	Forward
61	TAGTTTGATGTCGGGTGCTGAG	Reverse
62	GCGAATACCGGTCTTTTACGC	Forward
63	ATGCAAGCTCCGGATTGAGAG	Reverse

10 The primer set represented by SEQ ID NOs 58 and 59 (designated P5) was aimed at amplifying a 149 base pair region of SEQ ID NO 3, the primer set represented by SEQ ID NOS 60 and 61 (designated P8) was designed to amplify a 104 base pair region of SEQ ID NO 6 above, and the primer pair represented by
 15 SEQ ID NO 62 and 63 (designated P17) was designed to amplify a 130 base pair region of SEQ ID NO 10 above.

Thermocycling was carried out using a CFX96 Touch™ Real-Time PCR Detection System from Bio-rad. Initial denaturation was performed at 95 °C for 3 minutes; amplification was
 20 performed using 40 cycles of denaturation at 95°C for 5 seconds followed by 60°C for 30 seconds (plate read post each amplification).

All of the primer sets amplified Gd DNA with good efficiency as set out in Table 6, which shows the average Cq
 25 values of three replicates of the amplification, and quantitatively as illustrated by Figure 5. The percentage

efficiency was calculated using the formula $\%E = [10^{(-1/\text{slope})}] - 1 \times 100$.

Table 6

Log Dilutions	P5 (SEQ ID NOs 58 +59)	P8 (SEQ ID Nos 60 +61)	P17 (SEQ ID NOs 62 + 63)	ng/ μ l
-1	19.23	19.68	19.96	12
-2	22.75	22.55	23.39	1.2
-3	26.19	26.10	26.78	0.12
-4	30.23	29.97	30.75	0.012
-5	33.64	32.85	33.96	0.0012
-6	36.15	36.26	37.04	0.00012
-7	NA	NA	NA	
Slope	-3.4659	-3.3613	-3.4594	
%Efficiency	94.32	98.38	94.57	

5 The quantitative amplification was carried out in the presence of genomic plant DNA in order to determine whether there was any cross reactivity. It was found that whilst there was cross reactivity with some plants species, the primer pairs P5 showed no cross -reactivity to wheat and barley
10 genomes, P8 showed no cross-reactivity to wheat barley and maize and P17 showed no cross-reactivity to wheat and maize genomes making these potentially suitable primer sets for detecting Gd in crop species.

15 To ensure that primer efficiency and robustness would be maintained in the presence of plant genomic DNA, the above QPCR examples above were repeated but in this case, the composition was varied in that 6 μ L SDW (sterile distilled water) was used together with 1 μ L relevant Gd dilution DNA template and 1 μ L plant genomic DNA template. For instance, Gd DNA (92ng/ μ l) was
20 serially diluted from 10^{-1} to 10^{-7} with either wheat DNA (70.6ng/ μ l) or maize DNA (111ng/ μ l) and amplification reactions run as described above.

Representative results are shown in Figure 6(A) and (C) and in Table 7.

Table 7

Gd+Plant DNA QPCR Std. curve			
Log dilutions	Cq Value from QPCR run		ng/μl in reaction
	P5(Seq ID 3) <u>Wheat</u>	P17(Seq ID 10) <u>Maize</u>	
-1	21.47	21.76	9.2
-2	24.56	25.11	9.2
-3	27.69	28.13	0.92
-4	31.38	32.08	0.092
-5	34.89	35.32	0.0092
-6	37.55	37.80	0.00092
-7	N/A	N/A	0.000092
AzGd DNA (0.01)	24.20	25.00	
Plant DNA	N/A	NA	
NTC	NA	NA	
Slope	-3.2887	-3.2794	
%Efficiency	101.41	101.81	

5 It appears that the primers will maintain efficiency in the presence of plant genomes and thus may form the basis of a detection kit.

Results were confirmed by carrying out melt analysis post amplification the denaturation curve (Melt curve) analysis was performed from 60°C to 95°C with 0.5°C increment 5seconds/step followed by plate read after each increment.

Representative examples of the results are shown in Figure 6(B) and 6(E). Clear melt curves are visible for amplified Gd DNA, without plant genomic DNA.

15 Example 6

Plasmid Detection

Plasmid DNA extraction from Gd(IMI504853) was performed using Qiagen mini prep kit (Cat. No. 69104). The low copy number plasmid extraction protocol was followed using 5ml and 10ml 48hour bacterial culture. The extracted plasmid was run on 1%

agarose gel flanked by a 1kb hyper ladder plus (Figure 7) and imaged using Bio-rad ChemiDoc™.

Alongside the plasmid DNA, genomic DNA of Gd(IMI504853) was also included on lane-1. The results, shown in Figure 7
5 indicate the presence of a single plasmid of about 17.5Kb in size, which is smaller than that reported previously for plasmids found in PAL5.

The plasmid DNA was sequenced and a primer was designed using Primer3 (Untergasser et al. (2012) Primer3 - new
10 capabilities and interfaces. Nucleic Acids Research 40(15):e115; Koressaar and Remm (2007) Enhancements and modifications of primer design program Primer3 Bioinformatics 23(10):1289-91) to cover the start and end sites of linear sequence data (P_End_Fw - CCAAATCTCTGGAACGGGTA (SEQ ID NO 64). Sangar sequencing was
15 performed using this primer (SEQ ID NO 64) and the sequenced data was aligned to confirm the plasmid sequence was complete.

Since plasmid DNA in its natural form is circular and can form secondary and tertiary structures, this may impact on the accuracy of size measurements using agarose gels. To confirm
20 the results and also validate the sequencing of the plasmid DNA, a restriction map of plasmid was studied using NEBcutter (<http://nc2.neb.com/NEBcutter2/>). The restriction digestion will linearize the plasmid providing only a single conformational structure. Also, the restriction enzyme selection is
25 done after studying the sequence, thus allowing the plasmid sequence to be validated as well. In case of IMI504853_plasmid DNA, the NEB-cutter showed the restriction enzyme EcoRI to digest the plasmid DNA at 3864-9461bp and 9462-3863bp producing a DNA fragment of 5598bp and 11968bp. Both the size can be
30 studied using a 1Kb Hyper ladder plus (Bioline cat no - BIO-33069) available in the lab, removing the limitation of the reference ladder's maximum size detection as well.

Therefore, restriction digestion was performed on IMI504853 plasmid using double cutter EcoRI (Fisher, cat no -
35 10819360) as per supplier's protocol. Post restriction digestion the products were run on 1% agarose gel until the bands were

resolved and imaged using Bio-rad ChemiDoc™. IMI504853 plasmid DNA when restricted with EcoRI produced two fragments (1. ~12Kb and 2. ~5.6Kb) of DNA of predicted size (Figure 8).

This validated the sequencing data in terms of both size and sequence. It may further provide an identification test or a confirmatory test in relation to a kit used in the identification of strains of the invention.

Example 7

Illustration of Activity of IMI504853

A field trial was designed to test Gd (IMI504853) as a bio-fertilizer using wheat (cultivar Mulika). Two plots of Gd treated and control (untreated) respectively were planted. Post germination the young wheat seedlings at 10-12 day of growth were sampled and tested for Gd presence using the primer G (seq ID 26 & 27) representing the DNA seq ID 7. The Gd presence was detected when PCR was resolved on a 1% agarose gel with respective negative and positive controls (Fig 9) confirming that in real world condition the designed kit works well.

The measurement of chlorophyll content i.e. "greenness" using a SPAD meter has been shown to correlate with over all plant health and crop yield. The crop at growth stage 35 and 61 were checked for its chlorophyll content using SPAD was found to be statistically significantly ($P = 0.001$) in Gd treated plots when compared to control plots (Fig 10). Interestingly, the SPAD showed a significant increase in the chlorophyll content from the wheat obtained from plots treated with Gd of the invention compared to untreated controls (fig 10). This indicates that Gd treated plots which have been confirmed to have the bacterium present using the diagnostics kit results in much healthier plants and potentially higher yield. The data from wheat field trial indicates the efficacy of Gd as a bio-fertiliser.

Example 8

Yield Benefits of use of IMI504853

Forage maize seed untreated and treated with a formulation comprising IMI504853 at a concentration of about $10^5 - 10^7$ cfu/ml was sown at two different locations, and fertilised at

different proportions of the recommended rate of nitrogen fertiliser, ranging from 0 to 175 Kg ha⁻¹ for each particular variety, region and availability of soil nitrogen. Yield results indicated an overall potential reduction in nitrogen fertiliser of between 40-95% without suffering any yield penalty. Average yield benefits across N levels led to an increase in yield of between 7% and 21%. Thus at full recommended N fertiliser rates IMI504853 treated crops could provide a yield benefit up to 1 t/ha in maize.

10 In another trial, spring wheat seed, either untreated or treated with a formulation comprising IMI504853 at a concentration of about 10⁵ - 10⁷ cfu/ml was sown in Spring. The crop was fertilised at different proportions of the recommended rate of nitrogen fertilizer ranging from 0 to 125 Kg ha⁻¹ based on variety, region and availability of soil nitrogen. Across all 15 fertilizer levels, IMI504853 treated crops showed an average yield increase of 15%, but at zero nitrogen fertilizer and full nitrogen fertilizer this increase was 20% and 10% respectively. The results indicate that for the IMI504853 treated crop, it is 20 possible to reduce N fertiliser application by up to 85% and still achieve the same yield as a fully fertilised crop.

Table 1

SEQ ID 1	Glutathione S-transferase (EC 2.5.1.18)	ATGACAAAATTATACTATTCTCCCGGCGCTTGCTCTTTGGCAGGGCATATTTTGCTCGAAGAGTTGGGAAGACC ATATGAGTTGAAATTGACGCCCGTTGGAGACGAAGGCACGGGAAGTGAAGAGTTTCTAAAAATAAACCCGCGAG GAAGGGTGCCTGTTTAAATTGATGGTGGGAAATAATTACCGAAAGCCCGCAATCTTATTTTATTTATCGAGT TCATTTTCAGACGGAAATTTCTGGCCAAAATCAGTTTGGAGCAAGCCCGCTGCTGGGAATGGTTTAACTGGTT ATCGAGCAATGTACACTCGGTTGCCCTATGGGCAGGTGTGGCGACCGGTTTCATTGATGATGAGCGTCAGT GGAATAATGTTATTTCAAAAGGGAAAAATAACCTTCATGAATTTAGTGATGTAATAGAAAAATAATATCTCCGGG AAAACGTGGTGTGGGTGAATCGTATTCATGCGTTGATCCGTATTTGTTTGTGTTTTTATTTCTTGGGGGAAAGC CATCGGATTGGATATGGAATCCTCTTTCCCGCGTGGTCGCTCATGCAGCGGGATGCTGGAGCGGTTGGCCG TTCAAAAACGCTTTACGGCAAGAAGGTTTGATCTCGTAA
SEQ ID 2	O-methyltransferase (EC 2.1.1.-)	TTGGATGCCCTCTCGTTTTCCTTGGGAGTCATCATGACCATTCCTCTCTTTTCGTCCGCAATTCACACCGCAGAT TCAACGTGCGCTTGATCGCCTTTATTCGGAGACACTCTCGCAAGATCCAGCGATACGCCAATTTGGCGCAAGCCA AAGGACTGACACATGACGGGCAACGCGGTTTCTACGAAGCCATGAAAGATGCCAGACTACCCGTTACGCCCAGAG TTCGGCGCCCTGCTCTATATTCTGGCACGCAGCACCCAGAGCCCCAACATATCATCGAAATTCGGCACGTCCTTCGG TGTTTCAACATTAATTTCTCGACGCGGCTTTACCGGACAAATGGCGGGGCCGACTGGTGACCTCGGAACTTATCT CAGACAAAGCAGAAAAGGCTTCCGCCAATCTCGGGAGGCAGGACTGGCAGACCTCGTAGACATTCGCATCGGA GATGCCCCGCCACGTTATCGCGTGATCTTCCTGAGTCGATCGATCTGATCCTGTGATCGGACTAAGGACT CTACCTCGATCTCTTACTCCTGCTGGAACCTGCATTACGAAAAGGTGGCCTGGTGATCAGCGATCGCGCCGATC TCGATGGTGACGACGGCGGTGCGGCAGCAGCCTACCTTACCTATCTGACAACCCCGGCTAACGGATATCGCATC GCCGGCATCACTACACAGGCGTTGGGACAAAACCTTCGCTCACGATGTGGCGGTGGCCACCTGA

SEQ ID 3	Transcriptional regulator, TetR family	GTGGGAATAGCCACGCTCTACCAATACTTTGAGAACAAAGGAGAGCGTTGTGCGGGCACTTAGTCGTGCGGTACG GGAACACTGCTCCATGATGTTGCGTCAATTACTCGAAACCGCTTGTTCGTTGCCACTTTCGAGGGTGTGCGCT GTCGTGCTCGTCCGTGCGGTGAAGCGGACAAGAGCCGTCCATCGCTTACGGTCCGGCTTGATCGGTTGGAGGAG GCTCTTTCTTTGGAAGCGGATCATCTGCTGGTAGCGGCTGAGCTTTCACGGTTGTTGCGTCGTTCCCTCAAGTG CCAGGGAATTAATTCAGGAGAACACTGCATAAATTC'TGGCAGATGATCTGTGTACGATAACAGGGGCACTTATTG ACGCTGCACACAATCGACAGATAACCTATATCGATGACTTGCTAATTGACCGTATTACGGGAAGGCTGGTCGCGATT ATTGAGCGCGCTTTAA
SEQ ID 4	RNA polymerase ECF-type sigma factor :: RNA polymerase ECF-type sigma factor	GTGGGACAGCCGGACAAAATATTTGAGAGCTTTTCGCGATACATCGCACCGATCTTGTGCGTTTCGCCAGAGGTAT CATGAGAGATGATAGTCTGCGCGAAGATGTTGTACAGGATGCTTTTCTGCGGCTGACTACTGTAAACAGTGGCAC AGGACCGCGTTCTTTGCGATCCTCTGAATTACGTTTACCGCATTAATCGGAATCTGGCCTTTGACCGTTATATCGA CGACGGCAATTCGAGGCCGGATTGTTTGACCATGGGTAGATAGTTCTTCCGAAACAATCGAAGCGGATGCCCC TACACCGGAAGGTGAGGCTTCAGGGAAATCCGACATCGGGCAATCGCGCGCTATGGCGGAACCTGCCAGAAC GGACGTGCGTCGATTTGGAATGCATCTGTTCGACGGACGAAAGCTACGGGAAATAGCGGCTCATTTAGGTGTT TCTATTGGGATGGCCCACTCCCTTGTCGCGAGTGGGTATGGAGCATTGCCCGCAACGTCCTTTCCACACCTGAAAC CTGA
SEQ ID 5	FecR family protein, COG: Fe2+-dicitrate sensor, membrane component;	GTGAGCGAAGACTTCAATCCAACAACGGCGGTTGAGTGGAATAAGCCCTTTCAGAAAGAGCCTGACGATTCTGT GCTCAGAGAACGCTTTGAAGCATGGCTTGCTGCCGAGAGGATCACCGGAAAGAGTGGCAGGAACCTACGCAGG GACTTGAGAAATTTCCGCCAGATTGGCCCGCTTATCGTGAGAAATGGGTGCCCTTCATCAAGTGGGCAACAAAT ACTGCGTCAAAACAAGGTAGGCTCAAAGGAAGACCTGCTAATTTGTAGGTTTTCAGTTGCTGCATTTGCGGC TGCTGCTGCCGTTACATTGGTATGGTCCTCTGACCTTCTGCTTCGATTACAGGCCGATTACGCCACAGGCTCGG CAGAAAACGGCAACAGTCAGTCTCCCCGATGGTAGTGAATTGACCCCTCGCGCCCGGAAAGTGGCGTAAAAATGTCT TACTCTGTAGAGAAACGGGATATTCTGCTTTTAAAGGAGAGGCGCTCTTTCACGGTTCGACATGATATGGCGCG ACCTTTTGAGGTCCACACAGACAAAATTCACCGTAACGGACATCGGAACTATTTTGTACGTCAAGATGTCTCAGG

	SEQ ID 6 Ferrichrome-iron receptor GGAGGAAGAAGTCTCTGTCCGGGAAAGAGAGAGGTCCGGGTGCAGGATGTTTCCGGTGGATTTTCATAATCTCGAT GCCGGAACGTGGGAGCGGATTAGAACTGTAGGCAATGGAGTGAGCGTCACTCATGGGAGCGGCTCTCCGGAAGA TGTGGGCGCATGGTCAGCGGGGCAAAATTATTGCCAAGGAAAACAGCGTGTCCAGCGTCTGTGAAAAGGCTTAGGC CCTACTACCGGGGGTGTGTGTCTTTATGGTTCTTCCCTTTGGGAGAAAGTCACTCACTGGTGTATTATGACGCA AGTGACCCGGTTGGCGCATTTCCGGCGATTCGAGCCCGCGCATCATGCTCAGATGCATCAGGTTTTCGCCATGGCT GACAATATTGGCCGCACCGTAG ATGAAGGGTCCGGTTGCATTGCATTTCGCAATTGTGGCGGCTCATACGAATGGGAACGGATAAGGTGATGACGAT TGATGATAGAATGAAGCGGTGTGGCGGCAGGTGGCGTGGCTTATCGCGCTGGGTAGTACGACGTTTCTGAAATG CCGCTGTACGAAAAGCTATGGTGCAGAACCTTCCCAAAGTGTCTCGGGCCGTGAGATCATTTTCCATTCCGGCC CAATCTCTTGAAGATGGTCTCGCAAGGTTCGGACAGCAAAAGTGGGTGGCAGGTTTCTGTTGACGGAAAATCTTGC AAAATCTCTGACAAACGCACGGTGTTAGCGGTACGATGACATCTGCTCAGGCCCTCAATGCGATCCTGTCCGGGA CTGGCCTGACATACACGATCAGGGGTGGCCGAACCGTCTGTGACGAAAGCAGTAGCCAACATCACGCTTGGT CCGGTCCGTGTCCGAGGAACCTCGCGCGTCAGGATCCAACAGGGCCGGGTGTCCGCTACTTCGCCGAAAACAC AATGGTTGGTACAAAGACGGATACGCCCCATCACGGAAAATACCGAACTCAGTCTACGTCGTGACCAAGCAGTTGA TGACCGATCAGCAGCCCGAGAATATCCTACAGGCTTTGCGTTTACACTCCCGGCATCTACTCTGAAGCCGGAGGA ACGACAAAATCGCGGATCTGCCCAGAAATGACAAACATGGGCATTTATCAGCGTGGATTTCTCTCGAGCCAGTTCTGT GGATGGGTTGATGACGAAATTCGTATGCCGCCGCCGAGCCAAGCTTTCTGGACCGTATCGAGGCGCTCAACGGTC CAGCATCGGTGATGATGGCCAGACGACACCCCGAGGAATGGTCGGTATGAGCCTGAAGAAACCCACCGAAACG CCGCTGCATCAGGTTTCGCTAGGCTTCGGAAGCTGGGACGGTACGAGGCAACGTTGATGTGAGCGATAAGAT CACGCAGTCCGGTAATCTGCGCTATCGTATTCAGCCCATCGGAGTCACATCGGGCACTCAGGAAGACCCGAATTG ATTATCATCGGGTGGTGTACTTCCCTCAATCACGTGGGATATCGATCCCCAAGACTCGCCTCACTCTACTTGGT AGTTATATGTACACGCCCTGGCTCAGGGAGCACAGTGGTTATCCGGTCTCTCGGGACTCTTATTACAAATTCGGA AATTCACGAATCTCAGGAAGCACATTTATCGGAATACCTAGTTGGAACACTATGGGAGATAAGGTCGGGATGT
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	TCGAATATCAATTTAGTCATATAAATTTAATAAAATTTATTGAGTTCAATCAGACGTTTCGGCGTAGAGAAATCCAAC GTTTCATGAGTCAAAATATCACCGATGTAACACCTGTAGATGTTGAAGGAAAATGGACATATTTTATCCTTGGAA ACAAAATATGAAAAACAACACTGAGGTACTTGTACTCGCTTAGGGGGGGGTTTCTAACTGGTCCTGTACAAC ATACATGGGTCAATCGGTTCTGATTTCCGCAATTATGACTATCATATATACTGAGTCAATCGACGACGGTGCAGACA ATCGTTGTGCCCCACTCAGCACCCGACATCAAACTATTCGCCATGTATAAGTTTAACCTCCGCGAAGTGTGACGC CTTCGGCGGAATAAACCAGACTATAACTCGTTTCAGGAGGGCGTGTATTTTCAGGACCAGATAAAAATGGCAGC GCCGTGCCGTTCTTTGGGTGGACGCCAAGATTGGGTTAATTCACTTAATAAAAATTACAGTGTAAACGAACTTT TATGGAAAACGTGAGCACCCCGGTTAATAACACTGCTCCACACCCCTCAATCGGCCCTTCACCTGGAGAGCTGGTAT AATCTATAATTTTGACTTTGGGCTTGCCCCGTTACTTCAGTTACGCAACATCCTTTGTGCCACACAAGGAGGTACGG ATTGGCAGGGTAAGATTTTCGGCCCTTTGAGCGGAAAGCAACTCGAAAGCCGGGTTGAAGTATAAAGTTCCAAAC GAAGATATCCTCATAAACGGCATCAGCATTCGGAATTGATGAAGACCACCTATCTTATCAGTGATCTTGTTCACAC GGGCTTTAGCAGTGACGGGGAACGGTACGCTCGCAGGGTTTCGAGGTTTCGCCAGTTCGGAACATTAACAAAA ACCTCAAACTTGTCCGCTCTTACACATATGAGGATGTGCGGTTCAGAAAGAACAAATTTGGCCGTTAAATTCGGTC GATCCCCGTCACGCTAACATATGGAGCAAAGGTAAGCGAGAAATGAAAAATTCGTTCCCTCGAGTTCCTCGGAATAT GTTTAATATGTTTCTTGATTACACCTTCCACGACGCCCCCATTTGAAGGGTCTCGGCTTTAATGGAGGAATTCGCT ACACCGGTTTACCTATGCGGACTATGTGGAGTCTTACAAAAACGCCGGCGTATTATCTGTTTGACATTGGCGCA CACTATGATTTTGAGGAAATAATCCCTTCTCTCAAAAGTCTGCGTGCCCAAGTTGGCAATCTCAAAATTTGGCCAA TAAATATATATTAATTCGTGCAATACCGCCATATGTACTCTCGGTTATGCTCGAAAAGTTTACGGTAAACGTGA CGTATAGCTGGTGA
SEQ ID 7	Reverse transcriptase family protein

CCGGTACGTCGACGACATTAGACTGACCATAACAACACTGCACCAGGTATTGACCCAAAGAGAAGCTCAGGCGCGTG TAATGGCGTGGCTTGGGCAACTCCTCACGGGGAGCTGTCCGGGCTTGGAATTCTCCCCGGAGAAGACGTCAACT GCGTCGGTTGGAGGCGAGCAGATGCCGTGGTTCCGCAATCCGAAAGATGGAGCGCATCCAGACCGCGATTTC CGGCGGCTTCGATGCCAGTGGTGGCGAGGAGGTGATCCACGCGATCGAAGCCCTCGTCCGATCCAGCTAACGA TCAACAGCGTCGAAGAGTCGCCTACCCCTCCCGGCTTGAGAGCGGTACCCGATGTCAAAGACGAGACAGTCGGT CGTTTCGCTGCTGGTTCAGAAAAAACCTTTCGTTTCATTGAGGCCACTACTCGATGATCGAACCTTACATGGA GATTGCTGAATTCGGGGAGGAGACGTTCCGGCGCACCCGACTTTCGCAATCGGAGCTTGACGAGGAAGCACGCG CATTTGCGCTAATCCTAGTCGAACGGTGGATACTCGATCCTTCGAAATGTGCGGCTGCTGCGCGTCCGACTCGAC CTCGTGGCGTCCCGCCAACTCCTCAAGGAAGTACTGAAACTCTTTGAGCCCTATCTTGTGGGAAGATCAGGGC AATCACTAGCCGGCAAGTTGCATACTACTGCCCTCGCCGAGATATTTCGAGCAGGGCGACCGAGACGGGCTTCA TTGACGATCCAGAGTGCCCTTCCCGCTGCCGTGATCTGCCGGTTATAGATCTCTGCTTCTTGAGGCGCGCAGTA CGAGTGGCCCGGGCGAAGCCGAACGTGTCCCGTGGTATCTCGCGCAACAAGCACTGCTTTACATTGCGGTCCA CGATCCCCGGGCTATCCAAGATCGAGGAATTTCAAAGACCAATCGATCCTATTGGCGCCTCGTCTCATTTCTGA AAGCGGAACGCGACGTCTCTTCAGATCGCGAATTCGCAGTAGCCGCGGTGGTGAGCCGCGAGGTCTTCCTTTCCG AATGATCAGGCGGTGGATCTCGTCCGTCCGATGCTCACGCCAGAGCGGTTCGCCGAGGTGGCCGCGCGACAT AGAATTGCGCCGCGATCTCTTTCCGCCGCTCGACCGACACCTCACCGTTCCGGCAGGCATTGCCGAGGACTTGG GGGTCGCCGAATGGTCCATGTCAAGAGGAATGAGCTCTCTGCAAAAGCTATATCCAAGGCAAAAGGCCCTCTGAAT CCGCTACGCAATGAGATCGGCGTACTCAGTTTTCAGAGAAATTCATCTCCCATCTCCAAGAAGGAAAATTTGCC GGAAGTCGTACGCCGTCGACGACGAGATAGCGGTACAGCAAGTGGGCAAAATATGTCCGCGTCGAACGGGTGA TCTTCAGATCGGCCCCAGACAACGCCGACTTACCGGTCTATTTATCTGCTCCAGATGGGCGCCGGAATCTCAA CGCTGGCGCTTTCAGCTCGGTATTTACTTTCGCTTCATTCTTACTGCCAGAAATAGACTTCAGCCTTCCAGTTAG GCCGCCATCGTGAAGGAAGGTAACACATCTATCGGCCCTACCAGAACTCACTGGTTTCAGCGGCAATACGGCT TCTATAATGGGCATGAGGCCTTTCGGGGACGATTGGCTACCCATTTCCGAGTTCACTCAGGATCTTCTCTTCGAT	
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		CTGCTCACCTGGCCCCGGCTGCCGCACAAAGTAGCCCCGGATGTCGATCAGTTGTCCCTGGATGAAACGGCTGCTCG AATCCGGCGCAGCTCTCGTAGAAGCCACCAGCTGCGATTGGCCCCGGCTACAGGAACCCCTGATGCTCAAGATCGATG CACCTATTCCAGGTACCACATCGAAGGGGCGCCCGCTTCGGGCCCTGCGTCTCAGTCGATCATGCCCGAAGCA GAGGACTTCTCGGCTGCCGATCTGGAGATCGCGTCGCCGGCCCTTCGACGAAAGCACCGCAAAACATCTGTCCAC GGCATTTGGCGCGGTTGAGAAGATGTTGGATCTTCGGGAGACTCATTAAGCCAGCCAGCAAGCGTCTCGACTGGC TCATCCTACCAGAACTGTCTGTTCACCCCGGATGACGTTGCCACCCACCTCGTGGCCGTTCCGGCGGAGCGTTCAAG ACCGCGATCCTGGTCGGCATGGCCCTACGAAACAAGTCGTACGGGAGAGCCCGCTGATCAACTCGGCCCTCTCGGAT TATCCCGAGGATGGTCCGGGGCATGGGCCCTACAGACGGTGATCAGACGGCAGGGAAAAACAGCACCTCTCTCCGA TGGAAACAGAAGTACGTCAAACCGGTCGAACCTGATCACCGGATTCGCCGCCGTGCCAGTGGCTGGGTACGAA TGGTCGAACAATCCGGCCAAAGACGCACTTTGGCTCACCGCTCCATCTGCTACGATGCAACAGACCTGAAGCT GGCGAGCGATCTTCGTGATCGCTCAGACGTGTTTGGATCCAGCCCCCTGAATCTCGACGTCGGCACCTTTCGATC AGATGGCGCAGCGCTGCATTATCATATGTTCCAACCTCGTGTGATCGCGAAACAACGGAGCTTATGGGGGCAGC AATGCTCACGTTCCTAAAGGGGAGGCCCTATCAACGCCCAAGTGTTCATACCCATGGCCAGCCCCAGGCTACAAT TTCCTTTTTCGAGATCGACGATATCGAGGGCATGAAGCAGAGACACAAAGCTCGGCCGCTGGGAAGGAAGCGGGGT GGAAATATCCACCTGCCCGGCTGTCAAAGTCTGA
SEQ ID 8	DNA topology modulation protein	TTGATGCGCTTGTTCGTGACGGGGCCCAACTGGCAGTGGAATAACAAGCTGGCTGCAAAGTTGGCTCAAAGGGC AGCTATACCACTGTTCCCGCTCGATGAAATTTCATTGGGTTCCGCATCTCTCCGGGGATTGGCGCGCGATCCTG TTGAAACGCCCTGTCTATGCTCGGAGAGATTGTACAGCTCGATGCCCTGGTTCATCGAAGGCGTGCAGTTCAAATGG ACTGATATAGCGATAGAACGAGCAGACTGGATCGTCGTCCTCGATCCACAGTTGGCGGAACATCGCTCGTAT CCTGCGCCGTTTCGTCAATCGCCGATGCTTTTCTGGGGCGGGCACCGTGGAACGGTAAAGGCTCTATTGGAGG AGATGCGGTTGGTCAGCCGACTACTATGGTTCATGAACCGGGTATGCTGTTTCGAGAAGATTGGACAAATCGCCAGAC AAGCTCATCTGTCGTACATGACGACAAAGGGCGAAGCGGCTTTGACCGAGGCTGTATTTCGCGACTGCGTGA

SEQ ID 9	Cycloisomalttooli gosaccharide glucanotransfera se precursor (EC 2.4.1.-)	ATGGCATATTGGATCAGGCTCTCGCTGGCCGTGTGGCCGCCCGCATCAGCAACGTTGTAGCGAAGCCGCGTAAT GGCCCGCTATCTTTTCAACAACCATTTCTCTCGCTCTTACCCTCCCTTGGGGCGGGCATCCCTCCAAGGTCCGA TTGTTTCGCATGTGCGGGATGATCGGGCTTTCTACCAGGAGGCAATGTCGCATGATTTCCGTGGAACGTGACC CCACTAGCCGCTTGGACGGGAGGCCATGTGGATCTAGCGATATGTTGCGTGGCAAGTCGTGGCACGATTCA GAGCCAAGCGGTACACAGCATGTTGGCTGGGGCGGACAGACACTTCACTATCCCCTCACCGTTCCCAGTCTCC ATGCTCATGGGTATCAGTTGGCTATCGCGGCCCTGAACAATGGGGACAGCGGTCTGTACCCGGGACA GGCAGCACTTCCACGTCGCCCGCCGATGTGGCGTCAGGGCGCATCAACGTGGCCCGCAATGCCCTGGGAAGACAT CAACGAAGCATGGGTCCGACGGCGCGACGCTCGGCAACGCTCTCCGGGCCCGGGTGATGGATAATCTCAGCCAGT ATCACATCAACGGGTGCAATTTTACGACGTGCTGTGGCGACATGACGAGCCGTATTCTATCCGCCCGCGAGTGG CCGAACCTGGAAGGCGTAACGGTCACAAGGACCAATCTTCAGGCTTATATCAGCGCGGCATAGCCCGGCGCAT GGTGGCGCTCGCCTATAATCTCTGGAACGGAGCCTGGGGGACTATGAACTGTCAATCCGAAGGTCAACGGCGG CAATGGGGCTCTATGCTTCGTCCGGACAGAAACACCAACTGACCAACGGCGGGGCTGGCTGTCTCTGGGGGTGG TCGACCCGACCATATTGCTGAAATGAACCCGTTCAATGGCGACTGGGCCAGATGGCTAACCCAGCCAGATCCAGAA GACCATGTGGAATTTAGGATTCGACGCCCGCGCATCTGGATACGTTGGGTGACCCCTGGTGGTCAGCAATATGACG GCGAGGGCCATCCGTTACCTGCTTTAGGAACGATTCGGCAGACTTTGCGAATAATGTACAGGCTCAGACCCGGG GCACCAACTGACATCAACGCCGTTTCGGGTTGGAATACCAACCGACCTTTACCTACGCGGTACGGGACCCCAACCT GTATATCGAACCCTATCCCGAATTCGGAACAACGCCGGGCTACGATGATTCGGAAGCTTATGGGACATCAAAAC AGAAATATACGTCGCCCGCTGATGACGGCGTTTATCCGCAGCAGGTCAGAGCGGTTCCGTGAGCACGTCC TTTGGCCGTCAAGGGTGAGAGTGTGAAGGTTTGGACCCGACGTTAAAATCCGGATGCATAGCCAAATAACCTCGG CATTGAGTTGTGCTCGGCCAGATTGCGCTCAATGGAGGCTCCAATATTAATCTTGGTGATTTTGATCATCTGA TACCGGGGCCATATTTCCCCCGTCCGACCCCTTAAGATCGACGGTCCATTGCAGCAATATCTGGCGGATTACTAC AACTGGTGGGTGGAATGCGCGATCTGCTGCGGTGTCGGCGTCATCTCATCCAATGAGAGGGAGTCCATCCGGAA TGGAAACGGAGCCAGTATCGGCCAACCTTATGCCCAACCGGGAACCGTCTACTATCATCCCCCTGATACGCGCTG
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		GCATCGCTGGTGAATTGGCGCTCACAAACATGATCGGGTTGCATTATAATCGGATTGACGACCCTGACGGGCAAA AACAAATCCGACACCCCGGTGAACAACTGTTCGATCGAGATGGAATTTCTGGGAAAAGAACACACCGGGGGCATTTGTA CTATAGCGGCCCGACATCAACCACGGCTTCCACAGCCCTCACCTATAGGCTGAAACGGAACGGTAGCGTGA TGTTTACGCTACCGACTCTCAAGACGGTGGCGCTTGTTTGGCTGGAAGGCACCAATTTACCCACTACGACCGAT TACACGATCGGTACGGCGCAGGATGTAGGGGTGGACAGCAAACTTCTGGACGAACGGCAGCGGAGAGGATGC TACCGGATATCGTGGCTGCTGTGGTCGCTCCGCACGCTGGGACAGCATCGATTTCCGAGCGGGTGTGTCAACGC TAACGATGGTAACCCGAAGCCAACTCGGCGGACTGGTCGAATTTCCCTGGATGCACCTGATGGACCAAGTCATC GCCGGTAATTATGTTCTCTGGCTAGCGGCCACGACAAACCACTCAATTACGCAGGCCAGTATTCGGGGACACA TACCGTCTTCGCTAAAAATTCCTGGTCGGAGATTACGCTGATATCCTGGAAGCCATAA
SEQ ID 10	Methyltransferase (EC 2.1.1.-)	ATGATGGCTAACGACAAATACCACTGAGGTGGTGGTGCATTTGCGGTAAGTCATCCCACTTGGCGCAAGTTTT TACTTTTAGTAACAGCAGTCAACTAGATACGATTGCTTCTACTATTCAATAAAGCGGTTTGGAGACTTATGAAG CTCCGACAACATAATAATTATCGAACTGATCAGGAGTTCGTCTGGTCTTATTTAGATGTGGAGCGGAATACC GGTCTTTTACGCTAGTCGCCCGCAGCAGCCCAACCCCTGATCCGCGTCTGCTCTTTTGAGCCGCTTCCGAGTAT TCGTGAACTTCTCAAGAGCAATATTGCTCTCAATCCGGAGCTTGCATCACGTATCGCTGTGAGCCCTGTCCGGGT TATCGAATGAACGGGGCACTTTCACCTTTTACGAAACGATCAACAAATCGTGGCTTTGTACGACGAGTTTCATCG CTTGAAAAAGCACATGCAGAGCGAATCGGCCGATTGTACGTGAGCGCACTATCGAGACCCGGACACTTGATGA ATTGGGAGAAACGCTCGGGAATGCGAGCGTTCCGTTTCGTCAAAATTGACGTTGAGGGACATGAGCATGCCGTTA TCTCCGGTGGCCGCCACTTTATCGCCAAAGCACCGCCCTTTTCTTACTCTCGAAGTCCCTGAGAGAGGCTAACACT TTGAGTCTGGACCAAGTTGGTGACCGAGTCCAACTACCTTGCCCTGGCAATGGCACCCCGACGAATTCGGGCAGTG CGAGCGTTTACGGTTTCATGACGACGCCCTGGAATCATCTTTTGGTCCCCCGGAAAAAGCGGAACGGCTATTTT CGCTCTGCCCGCGACTTGGCTTGCAAAATCGGGATCCGCTGA

Table 2

SEQ ID 11	Transcriptional repressor; asserted_pathway : PF01381 Peptidase S24-like::PF00717	ATGTCGAATTCCGAGCGCCCAATGCGCGATTGTTCGGACCTGGCAAAAAACCGACAAAATAGAGCCGATGGTTAT CAGGCTACGAGAAGTAGTGGATCGGACCGGAGGCGCGAAAGCTGTGGCCGCACGCACGGACATCCCTCTCAGCA CACTTTCAGTTACCTGTTCGGGTTCGGGAACCTCAAGCTTTCCTGTCGCGCGCAAGATCACGGAAGCCTGCGGTGTC AGTCTTGACTGGCTTCGGCAGGAGAGACGACCTGCGGCCCGGGAATTCGGCAATGCCCGCAGCGCGGTCC CGAGTCGGTCGAGTTTCTGAATTACGACGTCATTTCTCCCTTTGTCCATTTCAGTCCAATACGGGACAAACATTTCCGGCC TCGAAAACGAGAAATATCGATACCGCGGGATTTTCTCCCTTTGTCCATTTCAGTCCAATACGGGACAAACATTTCCGGCC GTCACGGCGGAAATGCGACAGCATGAATCCGATCATAGACGATGGAGACATTTCTTTAATAAGAACGGATGTGCA TACGCTCAACAAGTGGCAGCATCTATGCCCTGCGGGTAGAAAAACACCCCTTCTGGTCAGGCGTCTGATCCTCAAGA CCAACGGCAACGTCAGGTCATCAGCGAGAACCCGCGTTACCCGACCGAGGAACCTGAACGCCGAGGACGTTCCGC AGGATGGTCCAGGACGACGGCTTTCGGCCAGGATCATCGCCGGGTCTATCTGGCGCGCGGTAGCCTGATTC ATAG
SEQ ID 12	outer membrane heme receptor	ATGGGCATCGTCTCTTGCCCTGCCTCGTCGCGACCTCAATAAGTATGTTGGCGGTTTCCGCATCCTATGCTTG GGCGGACAAATAGCCCCGTGCGCCCCCAGGACGAAACAAACAGGCCAAATCGCGGCCGTTACATGCCCAGGGGACGC GCAAGCGGGCAGCGCCATCACCCAGCCAGGATGAAGCGGTGGCTGTCTGTGGGAACACGTTGAGACATCGCATGGG ATGGAGCAGAGCGTTACCCGTGCGACGATGGACAAGTTCTGTGGCGGGACCAAGTCCCTGCAGATTCTGTCTGGC CACGACACCGGGTGTCAATTTTGCCCTCGGACGACCCGTTTCGGCTGGATACATGGCGGAACACATTTTATATTC GCGGCTATTTCCAAAGCCAGTTGGGCATCACCCCTGGACGGTATCCCGTGGCGGATGCCAGTTTCATCAATTCC AACGGCCCTCGATATCAATCAGGCGGATCATCCAGAACAAATATCGGTGCGTCGACATGTCTGCAGGGTGGCGGTGC GCTCGATGTCTCCGTCAACCAACCTGGGTGGCGGCTGCAATATTTCACTCGATCCGCGCGACAAAGGCTG GTGGAGACATTTACAGACGTTTCGGCAGCAACACAGACCTATCGCACGTACGTGAGCGCCAGAGCGGCAAGCTC AATCCCAGCGGGACGAAATTCTATGCGTCTGACGCGGACCCGATGCCGGGAAATGGAAAGGCCCGGGGACCA GTTTCGAAACAGCAGGCGAATTTCAAGATCGTACAGCCGCTCGGGCGTTACGGAAAACTGTCCGGATTCTTCAATT

		ATTCCGAATTCGACCAGTATAATTACAGCGATTTGAGCCTGGAAATCATCCAGAAGCTCGGCCGGAAACGTGGGAT TATTTCTATCCGAACTACAAAAGCCGCGTATCAGGCTGCCGAGGGGATCTATCCCGCAGGCTATGCCAAGGTCCG AGATGCCATGGACGTCTCCTATTACGATGGTGCCAGGACCAGCGGAATTATCTTTCCGGCATCACGTCCACGA TCGACCTGACGTCCCGCTGCATCTGAAGACGGTGCTGTACGACCAGCAATCGCGGGGGACTACGAATGGACC AACCCCTATGTGTCGTCGCCCTCGGGCGGCCCATGATCCAGCAGGTCGGGCACACATCGATGACGCGCGTGGG CGGGATTGGCGGGTGCAGTACCAGATCGCCAATCATTCGCTTGAAACCGGGCTCTGGTACGAAAAACAACGGAT ATAGCTGGGCGCAACGGTACTACAACCAAGCCGCTTCTGGGGGAGGGTACGCCCCGAAAGCGCCACCGGACCGTAC AACGATCCGTTCCGCCACCGCATACGCCATGACCTTCAATACCAACAGTTTTCATATATTACCTGGAAGATTCTCTA CCGTATCTTGAAGACGCTGCGGGTGCAACGCGGGCTTCAAAATCCATGCTGACGACGACGTCGGGGCGGCGCATCCT ATAACAAATCCCGTCTATACGGGCCAGGACACCCCTGCCAGTGGCAGCCTGACCACCGCCAGCGCCTTCTTGCCG CATGTCAGCATCAACTGGAATTTCTGCCCCGGAACGAACCTGTTTTCGACTTCGCGGAGAACATGCGCGCATT CACCTATAATACATGGCAGAGCGGGAATGCATGGGAGTCAATGAGATGCCCCAGAACCTGAAGCCCCGAGACCA CCTTCAATTACGAGGTCGGTTATCGATATAAATCCCCGCTTCGTACGGGCCCTCGTCAATCTGTATCATATCGAT TACAGGAACCGGCTGGCCACCATCACCAACCGGCAGCCCTGGTGAACGCCCCACAATACCTATATCAACGTGGGGAA CATGGCGATCTGGGGTGCCGATGCCGGCGTGACGGTGCGGCCCGCTGCCGGGCCCTCGAGATCTTCAACAGCGGCCA GCTACAAACAAATCCACCTATGGGCAGGATGTATCCAGCGGGGGGTAAATTATCCCATCAGCGGCAAGCAGGAG GCCGGCTATCCGCAATGGATGTACAAGGCCAACGTCTCGTACAGGTATGGCAACGCGAAGGTCAACTTCAACGT CAACTATATGGGAAAGCGATACATCTCGTACATGAACGACGCCGCCGCTGAACGGGTATTGGCTGGCATCGCTGT CGGCGACGTATATCTTCAAAACCATTCGCCATCTCTCTCAGCTTGAATTCAATTTCCGGCGTCTACAACCTGTTC AACCAGGAATATATCGGCGGCATCGGCGGGTTCTCACTGTCCGGTGACACGCAGCAACTCTTTGCCGGCGCGCC ACGCCAGTTCTTCGGTACGCTGCACGCACGGTTCTAG
SEQ ID 13	Levansucrase	GTGACGGCGCGGTGCTGCTGCAATCTGAAGAGTTTCTCTCAGGAGGATGGAATGGCGCATGTACGCCG AAAAGTAGCCACGCTGAATATGGCGTTGGCCGGTCCCTGCTCATGGTGCTGGCGCGCAAGTGGCGTGGCGC

	AAGGGAATTTACAGCCGGCAGGAAGCCGGCGCATGGCGCACCGTCCGGGTGTGATGCCCTCGTGGCGGCCCGCTC TTCCCCGGGGGTGGTGGCCGGGGTGGCCGGGCTTCCCGCTGCCAGCATTCATACGCAGCAGCGGTATGACCC GCAGTCGGACTTTACCGCCCGCTGGACACGTGCCGACGCATTCAGATCAAGCGCATTCGGATGCGACGGTCG CGCCCGGCGAGAATTCCTGCCGGCGCAACTGACCATGCCGAACATCCCGCGGACTTCCCGGTGATCAATCCG GATGCTGGGTCTGGGATACCTGGACCCCTGATCGACAAGCACGCCGATCAGTTCAGCTATAACGGCTGGGAAGT CATTTTCTGCTGACGGCCGACCCCAATGCCGGATACGGTTTCGACGACCCGCCACGTGTCATGCCCGCATCGGCT TCTTCTATCGTCGCGCGGGTATTTCCCGCCAGCCGGCGCGGTGAATGGCGGCTGGACCTATGGCGGCCATCTC TTCCCCGACGGAGCCAGCGCGCAGGTCTACGCCGGGCCAGACCTACAGAACCGGCGGAATGGTCCGGTTCGTC GGTCTGATGCAGATACATGGCAATACCGTATCGGTCTTCTATACCGACGTGGCGTTCAACCGTGACGCCCAACG CCAACAACATCACCCCGCGCAGGCCATCATCACCCAGACCTTGGGCGGATCCACGCCGACTTCAACCATGTC TGGTTCACGGGCTTCACCGCCACACGCCGCTGTGAGCCCGACGGCGTGTATCAGAACGGTGCGCAGAA CGAATTCCTCAATTTCCGCGATCCGTTACCTTCGAGGACCCGAAGCATCCCGCGTGAACTACATGGTGTTCG AGGGCAATACCGGGGCCAGCGTGGCGTCGCCAACTGCACCGAGGCCGATCTGGGCTTCCGCCCGAACGATCCC AATGCCGAAACCCCTGCAGGAAGTCCGTGGATAGCGGGGCCCTATTACCAGAAAGGCCAATATCGGCCCTGGCCATCGC CACGGATTGACCCCTGTCGAAATGGAAGTTCCCTGTCCCGCTGATTTCCGGCCAACTGCGGTCAATGACCCAGACCG AACGGCCCGCAGGTGTACCTCCATAACGGAAAAATACTATATCTTACCATCAGCCACCGCACGACCTTCGCGGCC GGTGTGATGGACCGGACGGCGTCTACGGCTTCGTGGGTGACGGCATCCGCACTTCCAGCCGATGAACATA TGGCAGCGGCTGACGATGGCAATCCGACCGACCTCAACACGGCGGCGAGGCACGGATTTTCGATCCAGCCCGG ACCAGAACCCCGGGCCCTTCCAGTCCATATTCGCACCTACGTATGCCGGGGGACTGGTTGAATCGTTCATCGAC ACGGTGGAAACCGTCGCGGGGGTACCCCTGGCGCCACCGGTCCGGGTGCGCATCGCCAGAACCGGTCCGCGGT CGACCTGCGGTACGGCAATGGCGGCCCTGGGCGGCTATGGCGATATTCCGGCCAAACCGCGCGGACGTGAACATCG CCGGCTTCATCCAGGATCTGTTCGGCCAGCCACGTTCGGGTCTGGCGGCGCAGGCGTCCACCAACAATGCCCCAG GTGCTGGCGCAGGTTCGCCAATTCCTGAACCACTAA	
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Claims

1. A strain of *Gluconacetobacter diazotrophicus* (Gd) characterised by the presence of at least one nucleic acid sequence selected from SEQ ID NOS 1-10 or variants or paralogues thereof and/or the presence of a single plasmid of about 17566 bp in size.
2. A strain according to claim 1 which is characterized by at least one nucleic acid of SEQ ID NOS 1 -10.
3. A strain according to claim 1 or claim 2 which is characterized by at least 3 different nucleic acid sequences of SEQ ID NOS 1-10.
4. A strain according to claim 3 which is characterized by at least 5 different nucleic acid sequences of SEQ ID NOS 1-10.
5. A strain according to claim 4 which is characterised by at least 8 different nucleic acid sequences of SEQ ID NOS 1-10.
6. A strain according to claim 5 which is characterised by the presence of all nucleic acid sequences of SEQ ID NOS 1-10.
7. A strain according to claim 1 which is characterised by the presence of a single plasmid of about 17566 bp in size.
8. A strain according to claim 8 wherein the plasmid is restricted into two fragments by the restriction enzyme EcoRI, wherein the fragments are about 12 and 5.6 kb in size.
9. A strain according to claim 7 or claim 8 wherein the plasmid lacks SEQ ID No. 65, SEQ ID No 66, SEQ ID No 67 and/or SEQ ID No 68.

10. A strain according to any one of the preceding claims which is IMI504853.

11. An agricultural composition comprising a strain according to any one of the preceding claims in combination with an agriculturally acceptable carrier.

12. An agricultural composition according to claim 11 which comprises a seed coating.

13. A method for enhancing the nitrogen-fixing ability of a plant, said method comprising administering to the plant or to the environment thereof, a strain of Gd according to any one of claims 1 to 10 a composition according to claim 11 or claim 12, so that the Gd colonises plants.

14. A method according to claim 13 wherein the strain is administered to a growth medium of the plant, to the surface of a seed, or to a wound of the plant.

15. A seed having a coating thereon, wherein the coating comprises a strain of Gd according to any one of claims 1 to 10.

16. A plant or seed having a strain of Gd according to any one of claims 1 to 10 colonised therein.

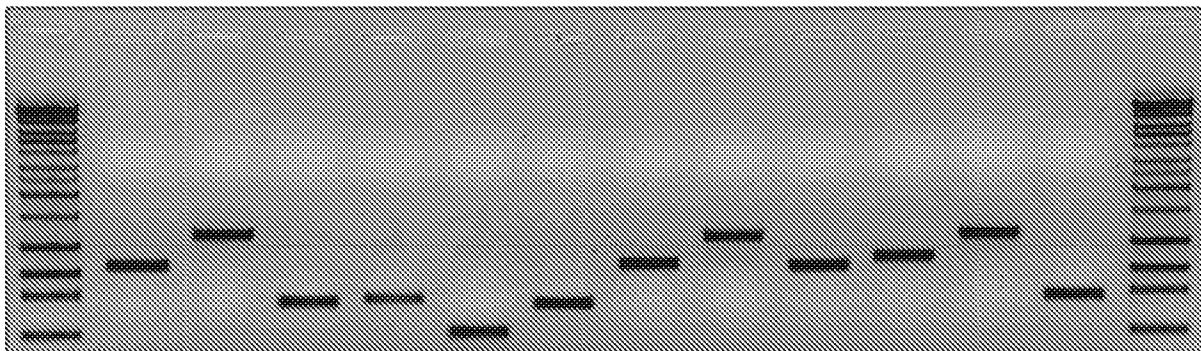
17. A seed or progeny of a plant or seed according to claim 16 having a strain of Gd according to any one of claims 1 to 10 located intracellularly in said seed.

18. A method for producing a plant product, said method comprising growing a plant according to claim 16 and obtaining the product therefrom.

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A

Figure 1



B

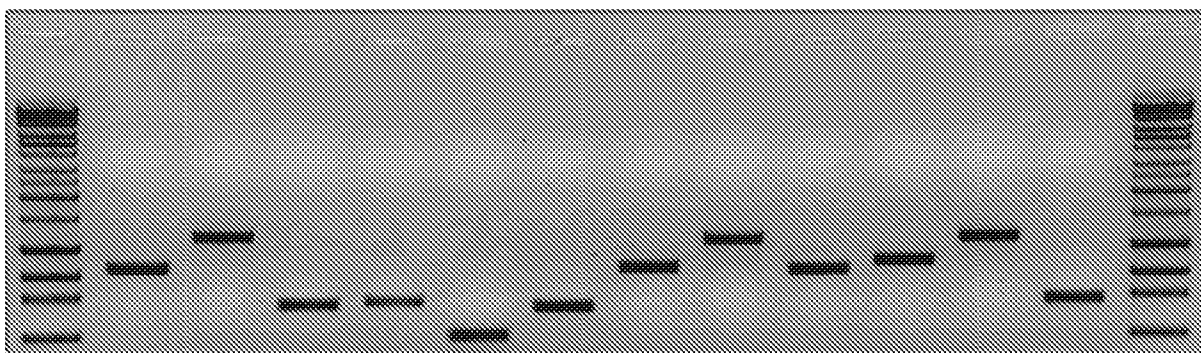


Figure 2

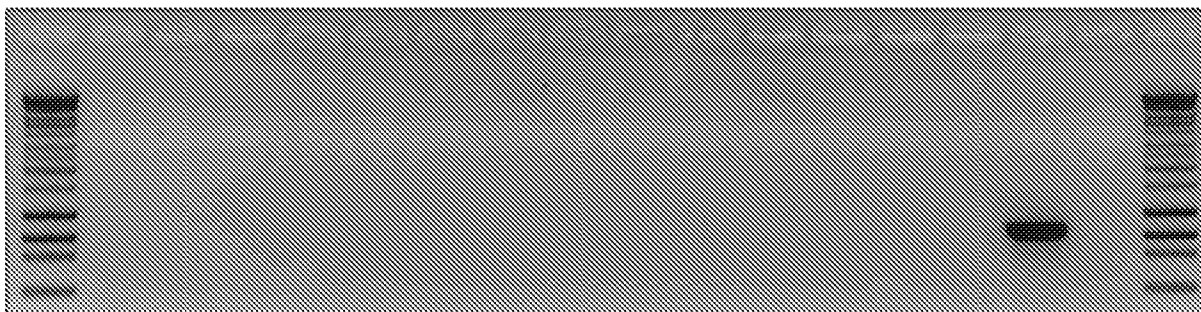


Figure 3

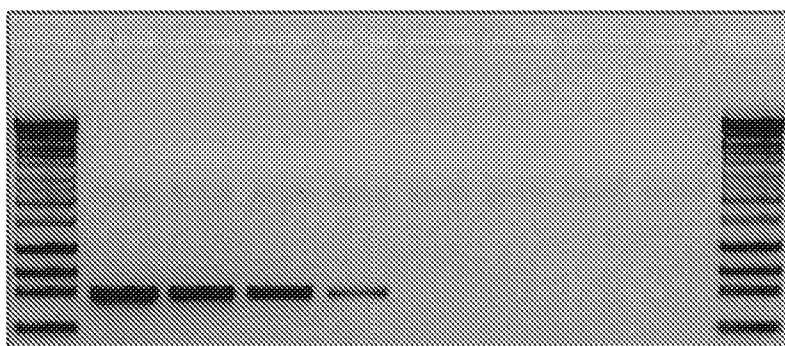


Figure 4

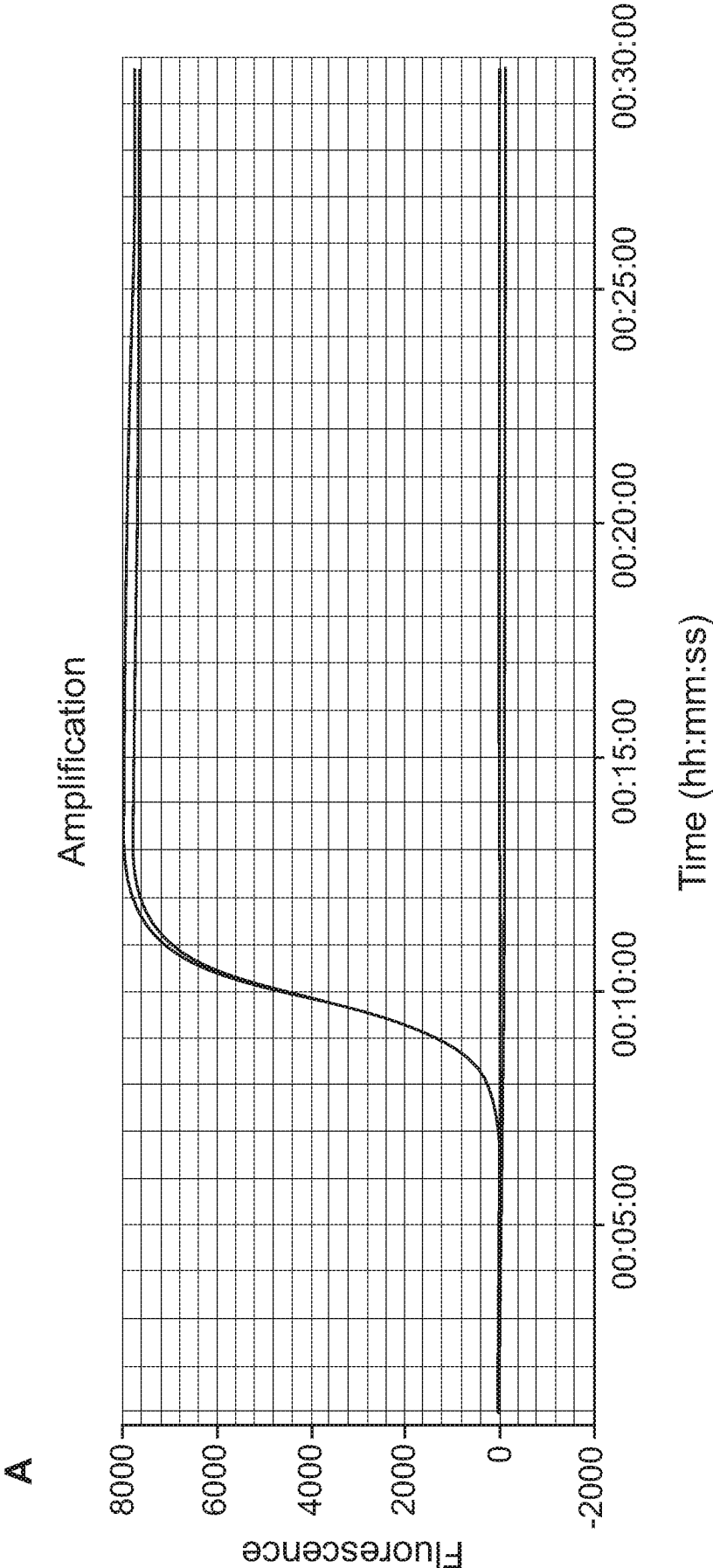
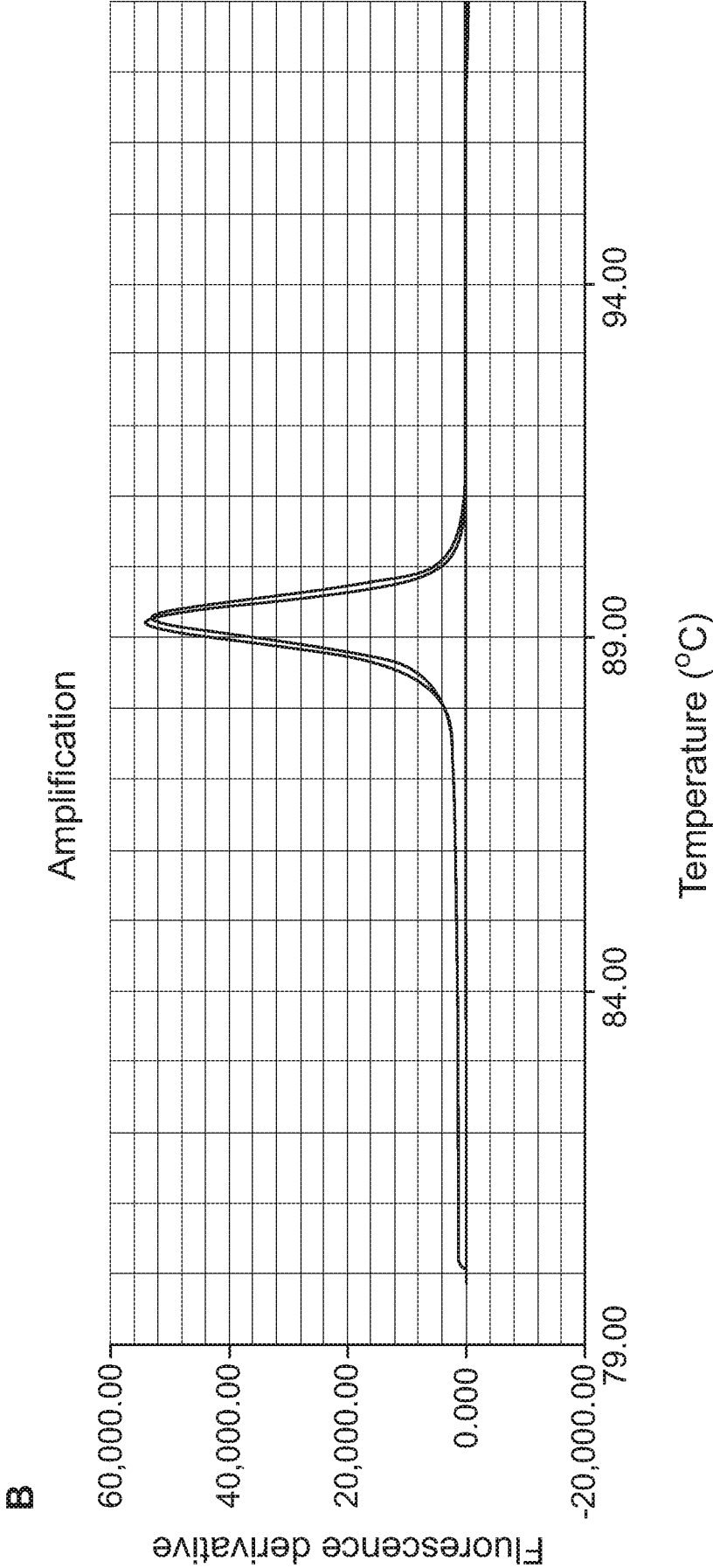
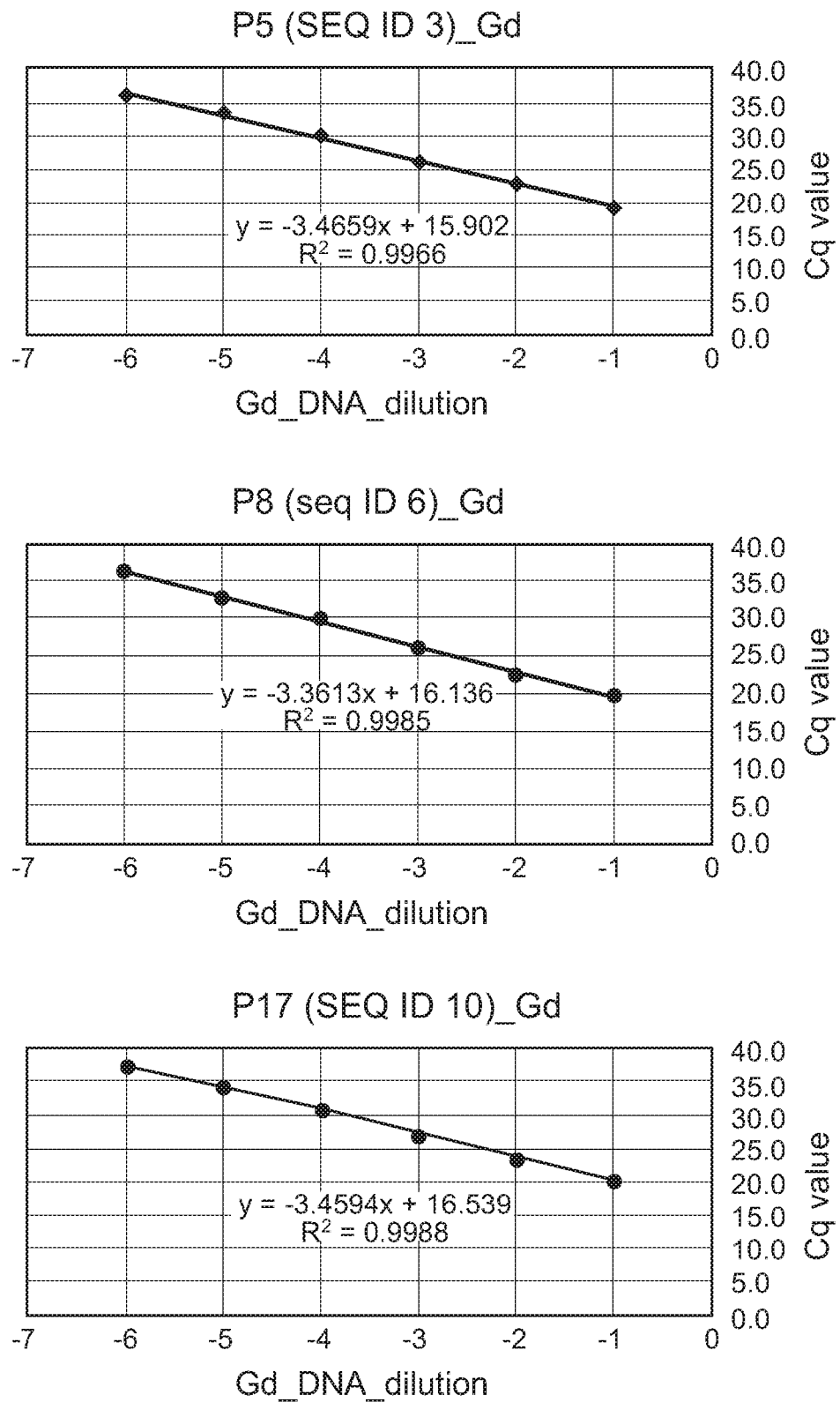


Figure 4 continued



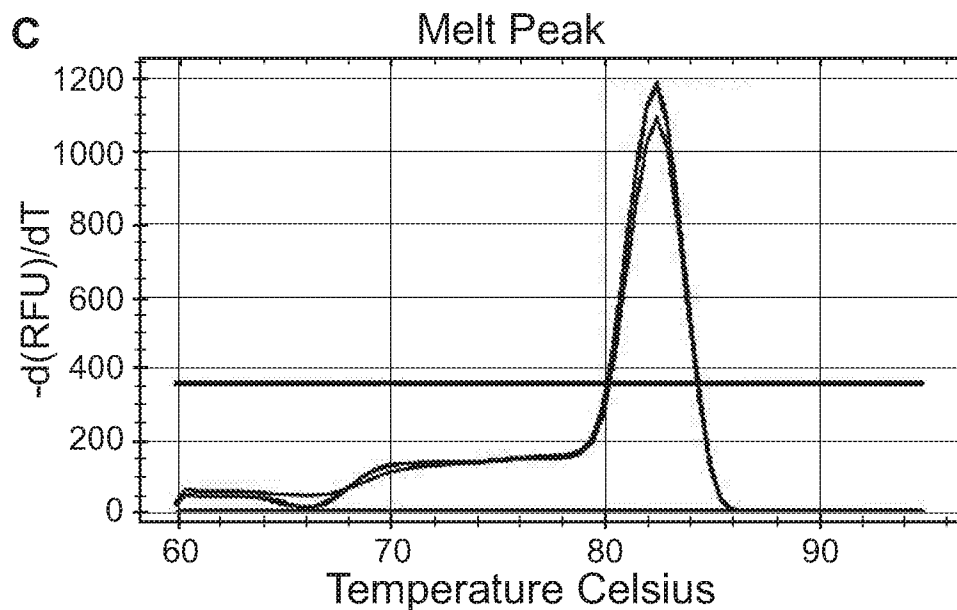
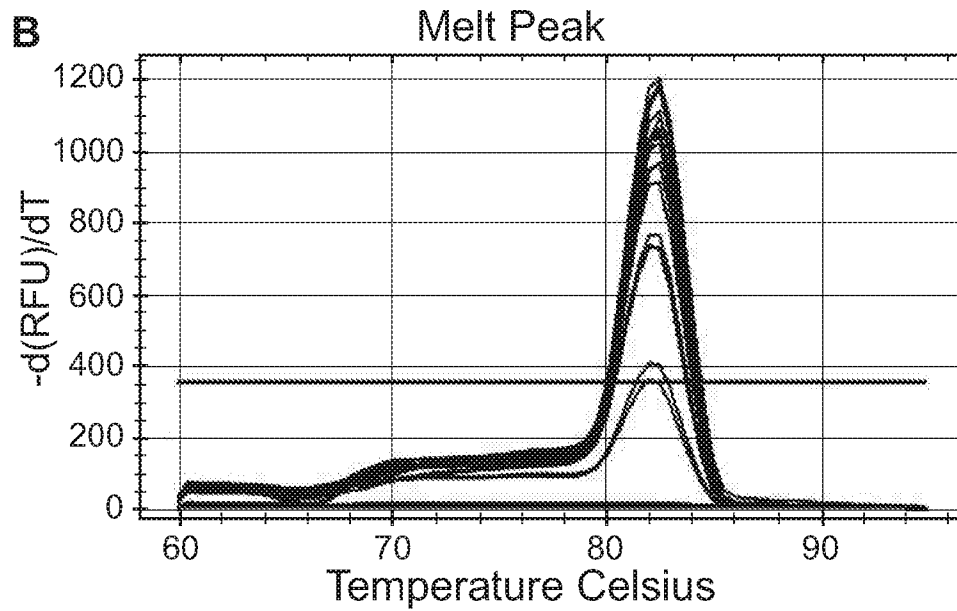
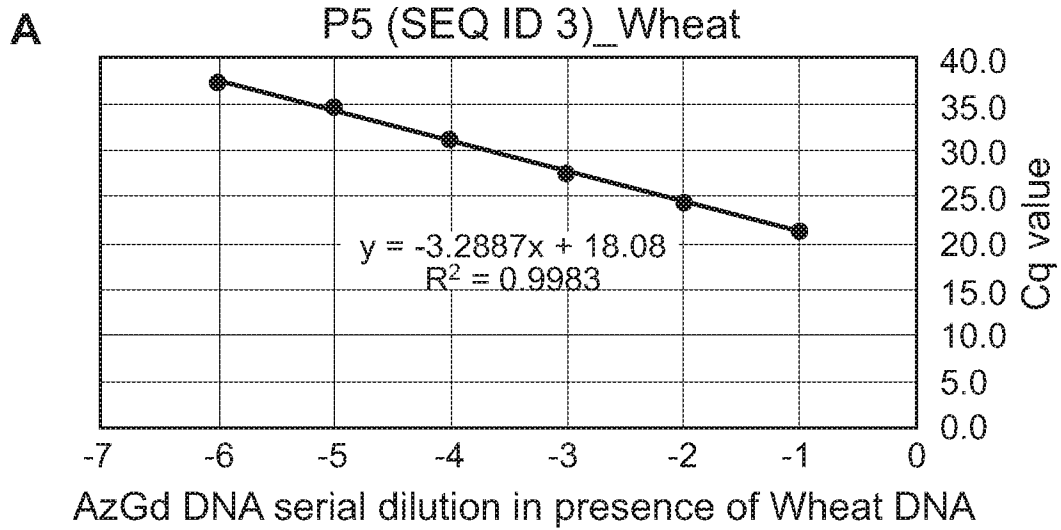
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Figure 5



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Figure 6



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Figure 6 continued

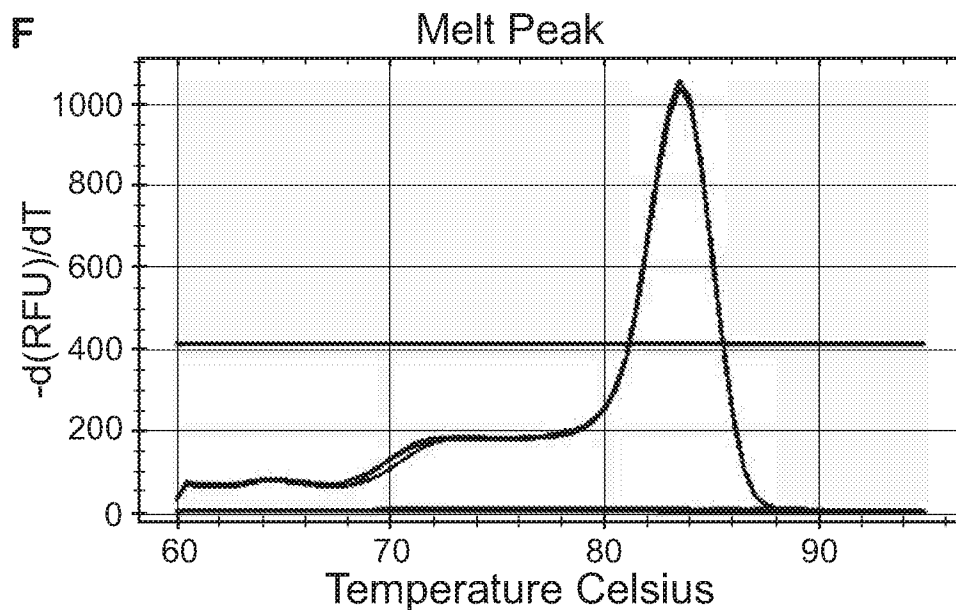
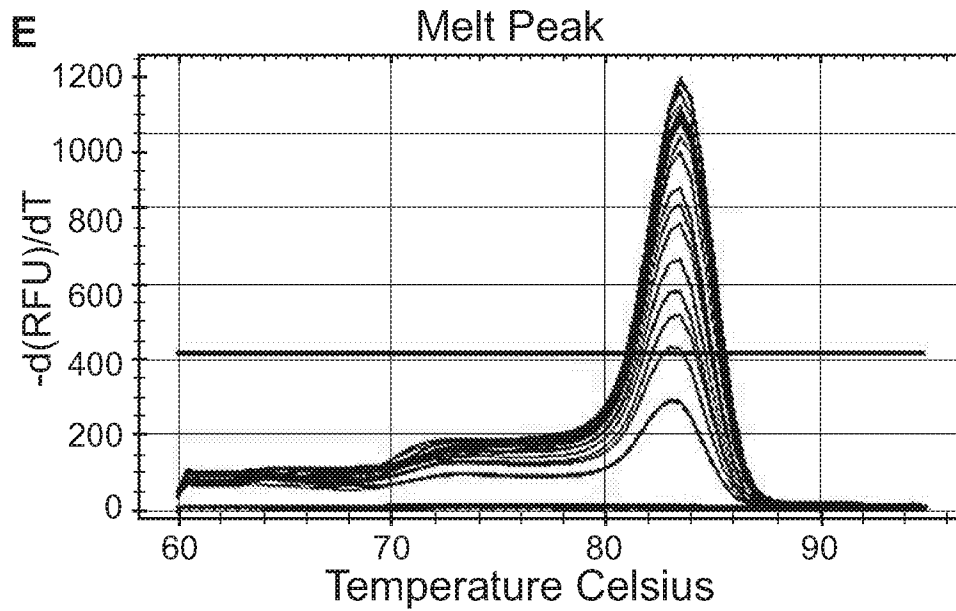
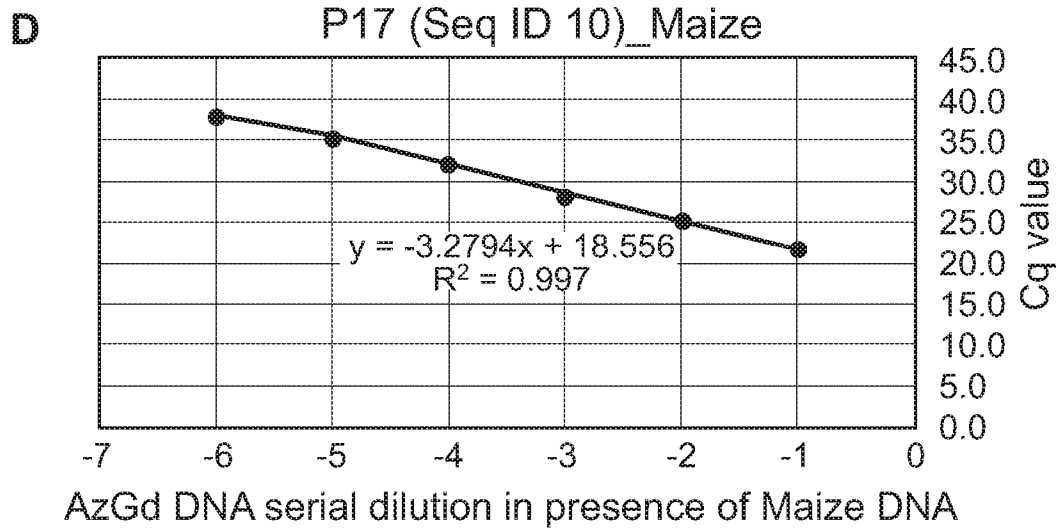
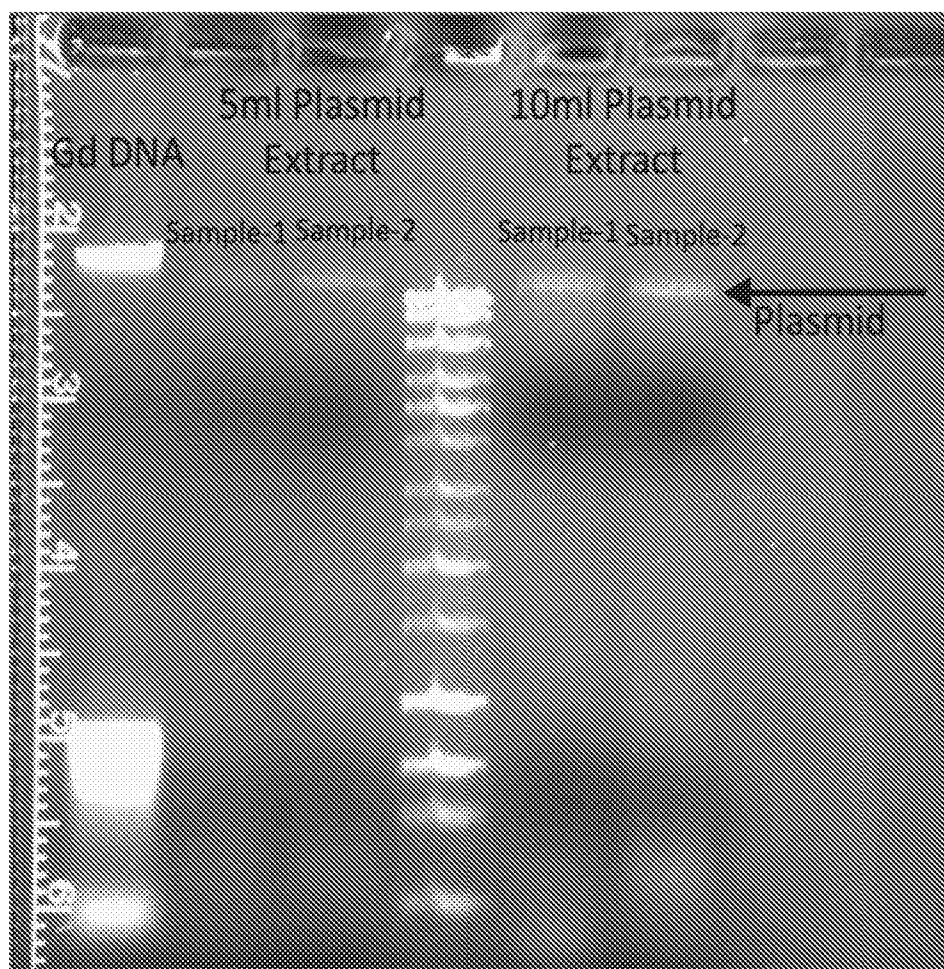


Figure 7



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Figure 8

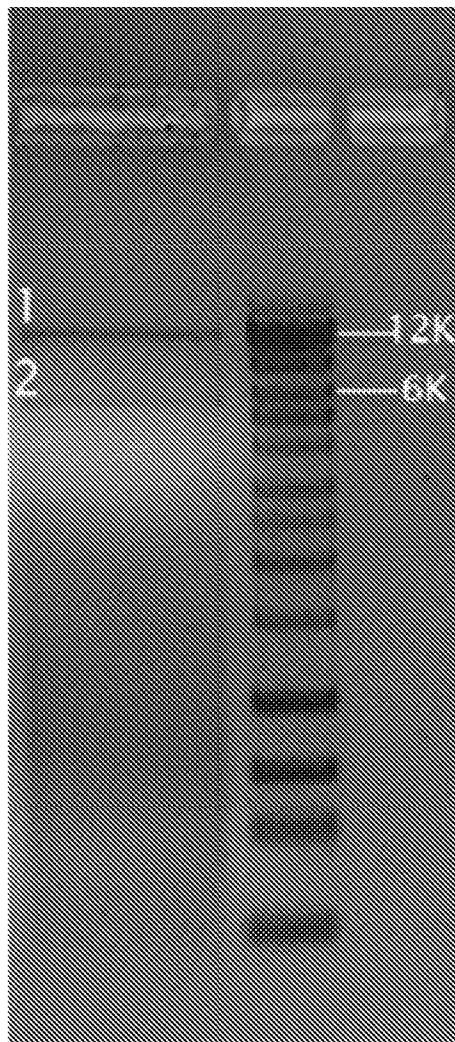


Figure 9

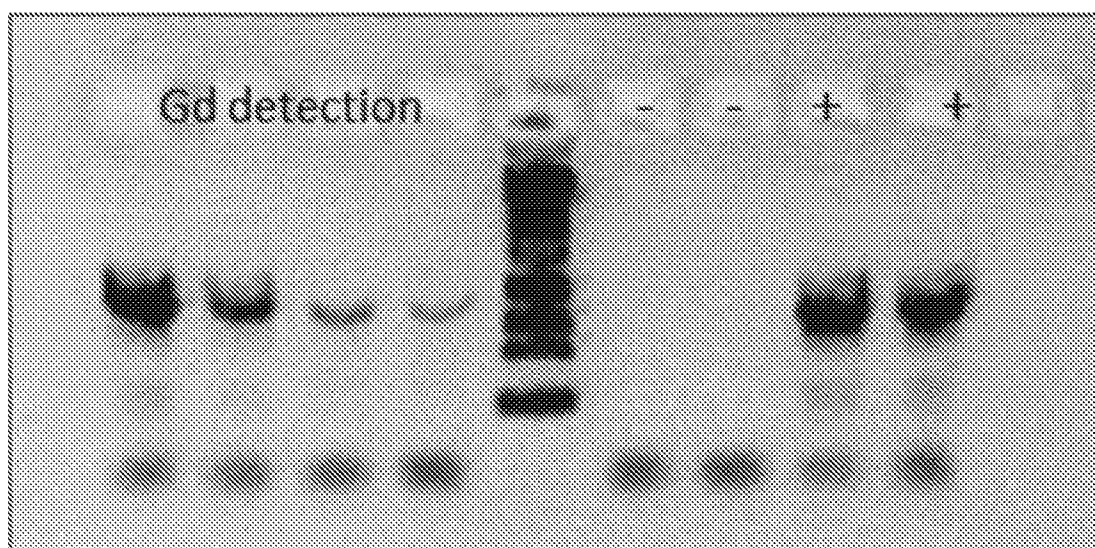
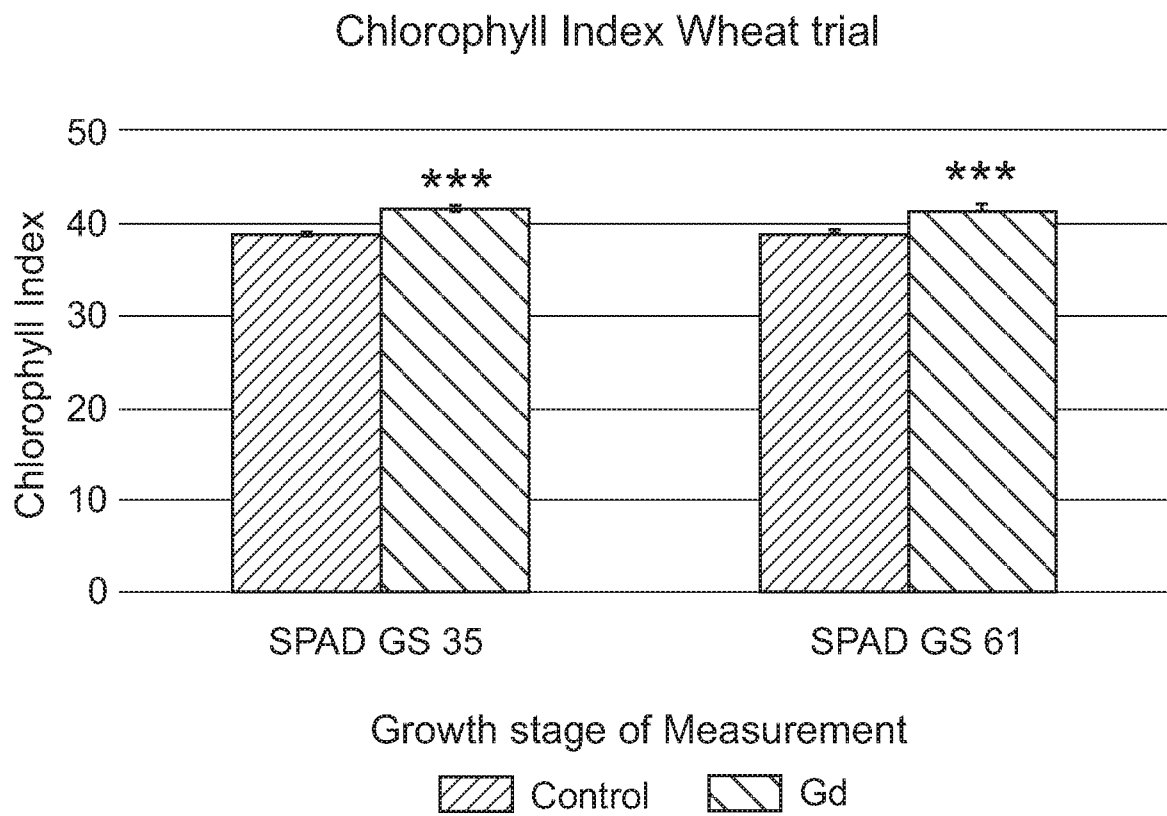


Figure 10



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/052289

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/20 A01N63/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/022517 A1 (SAINT MARY S UNIVERSITY [CA]; VESSEY J KEVIN [CA]; FEI HOUMAN [CA]; AB) 4 March 2010 (2010-03-04) tables 1-3	1-18
X	WO 03/020014 A2 (UNIV NOTTINGHAM [GB]; COCKING EDWARD CHARLES DANIEL [GB]) 13 March 2003 (2003-03-13) page 18; claims 1,2,14,33 ----- -/-	1-18

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

2 November 2016

Date of mailing of the international search report

11/11/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Bucka, Alexander

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB2016/052289

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/052289

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JESUS CABALLERO-MELLADO ET AL: "Limited Genetic Diversity in the Endophytic Sugarcane Bacterium Acetobacter diazotrophicus", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 60, no. 5, May 1994 (1994-05), pages 1532-1537, XP55310914, US ISSN: 0099-2240 table 2	1-18
A	----- BERTALAN MARCELO ET AL: "Complete genome sequence of the sugarcane nitrogen-fixing endophyte Gluconacetobacter diazotrophicus Pa15", BMC GENOMICS, BIOMED CENTRAL LTD, LONDON, UK, vol. 10, no. 1, 23 September 2009 (2009-09-23), page 450, XP021062138, ISSN: 1471-2164, DOI: 10.1186/1471-2164-10-450 the whole document	1-18
A	----- ADRIANA GIONGO ET AL: "Two genome sequences of the same bacterial strain, Gluconacetobacter diazotrophicus PA1 5, suggest a new standard in genome sequence submission", STANDARDS IN GENOMIC SCIENCES, vol. 2, no. 3, 30 June 2010 (2010-06-30), pages 309-317, XP55311123, DOI: 10.4056/sigs.972221 the whole document	1-18
A	----- ADRIANO-ANAYA M L ET AL: "Hydrolytic enzyme activities in maize (Zea mays) and sorghum (Sorghum bicolor) roots inoculated with Gluconacetobacter diazotrophicus and Glomus intraradices", SOIL BIOLOGY AND BIOCHEMISTRY, PERGAMON, OXFORD, GB, vol. 38, no. 5, 9 September 2005 (2005-09-09), pages 879-886, XP027985983, ISSN: 0038-0717 [retrieved on 2006-05-01] the whole document ----- -/--	1-18

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/052289

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FUENTES-RAMIREZ L E ET AL: "Colonization of sugarcane by Acetobacter diazotrophicus is inhibited by high N-fertilization", FEMS MICROBIOLOGY ECOLOGY, ELSEVIER, NL, vol. 29, no. 2, 1 June 1999 (1999-06-01), pages 117-128, XP002246475, ISSN: 0168-6496, DOI: 10.1016/S0168-6496(98)00125-1 the whole document -----	1-18
A	N. ESKIN ET AL: "Research Progress and Perspectives of Nitrogen Fixing Bacterium, Gluconacetobacter diazotrophicus , in Monocot Plants", INTERNATIONAL JOURNAL OF AGRONOMY, vol. 60, no. 10, 7 May 2014 (2014-05-07), pages 1532-13, XP55218778, ISSN: 1687-8159, DOI: 10.1016/j.chemosphere.2006.07.067 table 7 -----	1-18
A	PEDRAZA ET AL: "Recent advances in nitrogen-fixing acetic acid bacteria", INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, ELSEVIER BV, NL, vol. 125, no. 1, 5 December 2007 (2007-12-05), pages 25-35, XP022709795, ISSN: 0168-1605, DOI: 10.1016/J.IJFOODMICRO.2007.11.079 [retrieved on 2007-12-05] table 1 -----	1-18
A	VERONICA MASSENA REIS ET AL: "Nitrogen fixing bacteria in the family Acetobacteraceae and their role in agriculture", JOURNAL OF BASIC MICROBIOLOGY, vol. 55, no. 8, 3 March 2015 (2015-03-03), pages 931-949, XP55311275, BERLIN, DE ISSN: 0233-111X, DOI: 10.1002/jobm.201400898 the whole document -----	1-18
X,P	WO 2016/016629 A1 (AZOTIC TECHNOLOGIES LTD [GB]) 4 February 2016 (2016-02-04) page 2 -----	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2016/052289

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010022517 A1	04-03-2010	CA 2735269 A1 US 2011225679 A1 WO 2010022517 A1	04-03-2010 15-09-2011 04-03-2010
WO 03020014 A2	13-03-2003	AT 399459 T AT 504204 T AU 2002321575 A1 CA 2458933 A1 EP 1422997 A2 EP 1714545 A1 ES 2309191 T3 US 2004235663 A1 US 2009106865 A1 WO 03020014 A2	15-07-2008 15-04-2011 18-03-2003 13-03-2003 02-06-2004 25-10-2006 16-12-2008 25-11-2004 23-04-2009 13-03-2003
WO 2016016629 A1	04-02-2016	NONE	