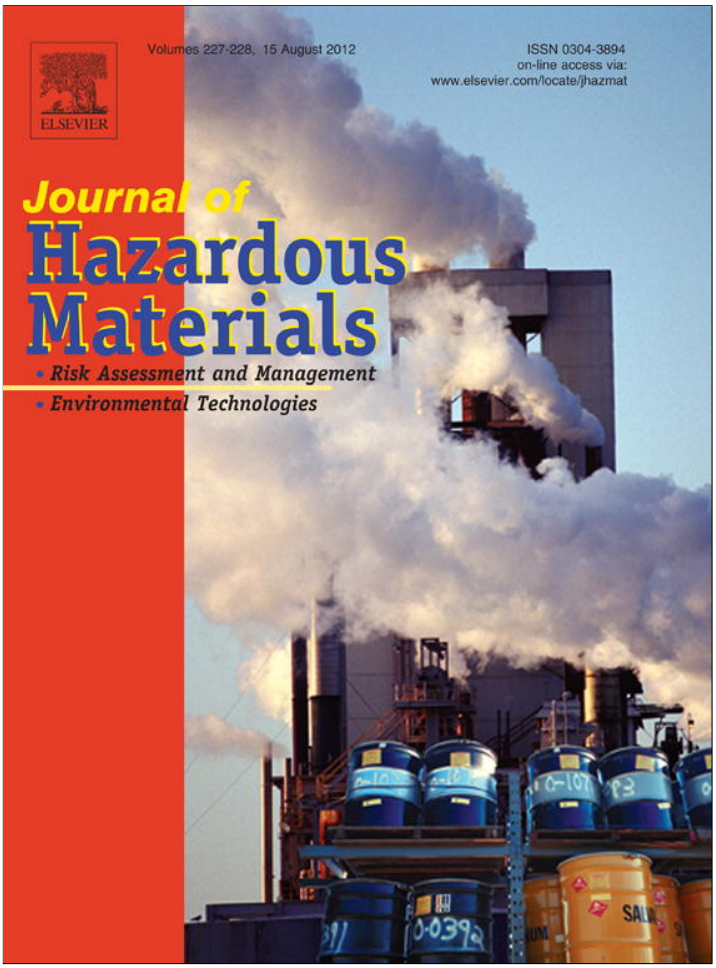

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Improved detection of microbial risk of releasing genetically modified bacteria in soil by using massive sequencing and antibiotic resistance selection

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HIGHLIGHTS

- GM bacteria/element diminished with no shift in structure of bacterial community.
- Abundance of antibiotic resistant bacteria and potential pathogens were altered.
- Potential risk of GM *Corynebacterium glutamicum* was less than GM *Escherichia coli.*
- Stability of GM plasmid may influence the potential risk of GM bacteria.

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ABSTRACT

High-throughput 16S rRNA gene-targeted pyrosequencing was used with commonly used risk assessment techniques to evaluate the potential microbial risk in soil after inoculating genetically modified (GM) *Corynebacterium glutamicum.* To verify the risk, reference experiments were conducted in parallel using well-defined and frequently used GM *Escherichia coli* and wild-type strains. The viable cell count showed that the number of GM bacteria in the soil was reduced to below the detection limit within 10 days, while the molecular indicator for GM plasmids was detected throughout the experiment by using quantitative real-time polymerase chain reactions. Subsequent pyrosequencing showed an insignificant influence of the GM bacteria and/or their GM plasmids on the structure of the soil bacterial community this was similar to non-GM wild-type strains. However, pyrosequencing combined with kanamycin-resistant bacteria selection uncovered a potential risk of GM bacteria on the soil bacterial community and pathogens. The results of the improved methodology showed that the microbial risk attributable to GM *C. glutamicum* was relatively lower than that attributable to the reference GM *E. coli.*

1. Introduction

The development of genetic engineering technology has given us the ability to grant particular traits to organisms and to enhance existing traits. This has drawn the attention of the microbiology industry, as such technology may advance conventional bio-processes with minimal cost. Despite a growing emphasis on the potential of this technology, genetically modified (GM) microorganisms are seldom used in the production of commercial commodities. This hesitation can be attributed chiefly to an inadequate knowledge of the potential risks associated with the deliberate and/or accidental release [1] of these microorganisms. The risks, as perceived by the general public, together with the fast-growing microbiology industry, have led international organizations to publish guidelines for the use of GM microorganisms [2,3] and governments to request thorough safety reviews before their commercialization [4].

Efforts to verify the environmental risks of releasing GM microorganisms have expanded as a result of the widespread application of genetic engineering methods. However, these assessments are commonly performed using techniques such as Cloning & Sanger sequencing and fingerprinting, which may be inadequate for detecting detailed changes in complex microbial communities, such as those present in the environment [5,6]. As a result, perspectives on environmental risks have become very diverse and inconsistent, hampering research efforts in the field. In contrast, advances in genetic engineering techniques have resulted in the creation of many more GM microorganisms with potential economic value [7]. Thus, methods for the qualitative analysis of biosafety are urgently required to support the fast-growing microbiology industry.

The development of gene-targeted pyrosequencing has made in-depth analysis of complex microbial communities feasible through the massive and parallel sequencing of 16S rRNA

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amplicons [8]. In particular, Bibby et al. [9] recently used this technique to monitor pathogens in environmental samples. Adapting pyrosequencing to risk assessments of GM bacteria may circumvent the technical issues, such as low resolution, narrow spectrum, that have been present in commonly used techniques. However, the applicability of this technique for evaluating environmental risk has yet to be examined.

In this study, we investigated the potential microbial risk of releasing commercial GM bacteria through evaluating frequently addressed risk criteria [10,11] using 16S rRNA gene-targeted pyrosequencing along with commonly used assessment methods. In addition, the spread of antibiotic resistance and proliferation of pathogens were investigated. GM *Corynebacterium glutamicum* containing a GM plasmid with a kanamycin resistant gene was used as the target GM bacteria, with well-defined GM *Escherichia coli* and wild-type strains used as references. To enhance the detection of changes in antibiotic resistance bacteria and pathogens, the pyrosequencing method was combined with antibiotic resistance selection. This improved detection of known bacterial pathogens in kanamycin-resistant (kr) communities isolated from GM bacteria inoculated soil.

2. Materials and methods

2.1. Bacterial inoculum

C. glutamicum ATCC 13032, *E. coli* TOP10, and their GM derivatives were used as the bacterial inocula. *C. glutamicum* and GM *C. glutamicum* were provided by CJ Cheiljedang Corp. (Seoul, South Korea). GM *C. glutamicum* contains pCJ1-TNAI_L469P plasmid with kr aminoglycoside 3′-phosphotransferase type 1 (*aphA1*) genes. *E. coli* was obtained from a TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, CA, USA), and GM *E. coli* was constructed by transforming pCR2.1-TOPO plasmid containing an *aphA1* gene. The pCR2.1-TOPO plasmid was prepared as follows: plasmid DNA was extracted from GM *C. glutamicum* using a QIAquick Spin Plasmid Miniprep kit (Qiagen Inc., Valencia, CA, USA). The *aphA1* regions were amplified using a polymerase chain reaction (PCR) with L-AI primers (CJ Cheiljedang Corp.) and a C1000 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR amplicons were inserted into pCR2.1-TOPO plasmid and then transformed into *E. coli* using a TOPO TA cloning kit. The volume of the PCR mix was 25 μL, and its constituents were as follows: 3.3 μL (10–15 ng) of plasmid DNA, 2.5 μL of 10 μM L-AI_F (5′-ATGATCGATCTCAAACAGTATGAGTTC-3′) and L-AI_R (5′ TCATCTTTTTAAAAGTCCCCAGTAGAG-3′) primers, 0.2 U of AccuPrime Taq Polymerase High Fidelity, 2.5 μL of AccuPrime PCR Buffer I (Invitrogen Corp.), and 14 μL of deionized water. The PCR conditions were as follows: denaturation at 94 °C for 3 min; 25 cycles of (i) 95 °C for 1 min, (ii) 54 °C for 30 s, and (iii) 72 °C for 2 min; and final extension at 72 °C for 2 min.

The *C. glutamicum* and *E. coli* were cultivated in 500 mL of Luria-Bertani (LB) broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in the dark for 24 h at 30 °C, with agitation (170 rpm). Their GM derivatives were cultivated in 500 mL of LB broth supplemented with kanamycin (50 μg/mL) under the same culturing conditions. After incubation, bacteria were washed three times using sterile 0.01 M phosphate buffered saline (PBS) and then re-suspended in 100 mL of fresh PBS.

2.2. Soil microcosms

Sub-surface soil samples (5–10 cm below the surface) were collected from ShinDongBang CP (Ansan-shi, Gyonggi-do, South Korea), where a GM *C. glutamicum* culturing facility is to be constructed (37°17′30.21″ × 126°53′26.84″). The soil was characterized as sandy loam consisting of 60.9% sand, 34.4% silt, and 4.7% clay, with a pH of 6.5 and an organic content of 3.9%. Particles greater than 2 mm in diameter were removed through filtering.

Five beakers, each containing 600 g of the filtered soil, were prepared, and four of them were inoculated with 180 mL of PBS that contain *C. glutamicum*, *E. coli*, GM *C. glutamicum*, or GM *E. coli*; the beakers were labeled WC, WE, GC, or GE, respectively. The remaining beaker was inoculated with only 180 mL of PBS as a negative control and labeled as CN. The microcosms were mixed, tightly sealed and then incubated in the dark for up to 94 days at 25 °C.

The bacterial inocula contained 1/10 of the culturable bacteria present in 600 g of the filtered soil. The bacterial populations in the samples were quantified using viable cell counts. For this, 10-fold serial dilutions were prepared using PBS. Then, 100 μL of the adequately diluted samples were spread onto LB agar and cultured in the dark for 3 days at 30 °C. The average number of colony forming units (CFUs) from three independent experiments was considered as the number of culturable bacteria in a sample.

2.3. Quantification of culturable kanamycin-resistant bacteria

The survival of GM bacteria in soil microcosms and their influence on kr bacterial populations were evaluated using viable cell counts. For this, kr bacterial populations in soil microcosms were analyzed at various times of interest (0, 3, 6, 10, 17, 23, 31, 38, and 94 days) following the culturing method described in Section 2.2 using LB agar supplemented with kanamycin (50 μg/mL) as culturing media. Sampling times were in shorter intervals at the earlier phase of the experiment (before day 10) to explore the decay kinetics of kr bacteria. After this period, the interval was fixed to 7 days to observe possible long-term changes in kr bacteria populations.

2.4. Quantification of the aphA1 gene

The persistence of GM plasmids (pCJ1-TNAI_L469P and pCR-2.1-TOPO) in soil microcosms was evaluated indirectly by quantifying the *aphA1* gene. For this, genomic DNA (gDNA) was extracted from soil microcosms at times of interest using a PowerSoil DNA Extraction Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). From the gDNA, *aphA1* copies were measured using qPCR with TaqMan probes. The volume of each reaction mix was 25 μL, containing 5 μL (10–15 ng) of gDNA, 3 μL of 10 μM Kana-98 forward (5′-GRGTCGGAATCGCAGACCG-3′) and reverse (5′-ATATTTTTGAAAAAGCCGTTTCTGTAAT-3′) primers, 2 μL of Probe (FAM 5′-CTGAACCAATCTGCCCACGGTGACA-3′ TAMRA), 12.5 μL of Master Mix, and 2.5 μL of deionized water. The thermal cycling conditions were as follows: initiation at 50 °C for 2 min, enzyme activation at 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. An Applied BioSystems 7500 Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) was used to conduct the PCR.

2.5. Shifts in the bacterial community

2.5.1. High-throughput 16S rRNA gene-targeted pyrosequencing

gDNA was extracted from the soil microcosms after 0, 3, 10, 38 and 94 days, as described in Section 2.4. Sampling times were chosen to investigate shifts in the soil bacterial community in the presence of viable GM bacteria, to observe potential re-habitation of GM bacteria, and to monitor long term changes in the bacterial community. To selectively gain gDNA from kr bacteria, soil microcosms sampled on day 10 were further incubated in the dark for 3 days at 30 °C on LB agar supplemented with kanamycin (50 μg/mL) prior to gDNA extraction. The 16S rRNA genes were amplified by performing PCR according to the method previously described by Lee et al. [12]. DNA barcodes are available in the supplementary

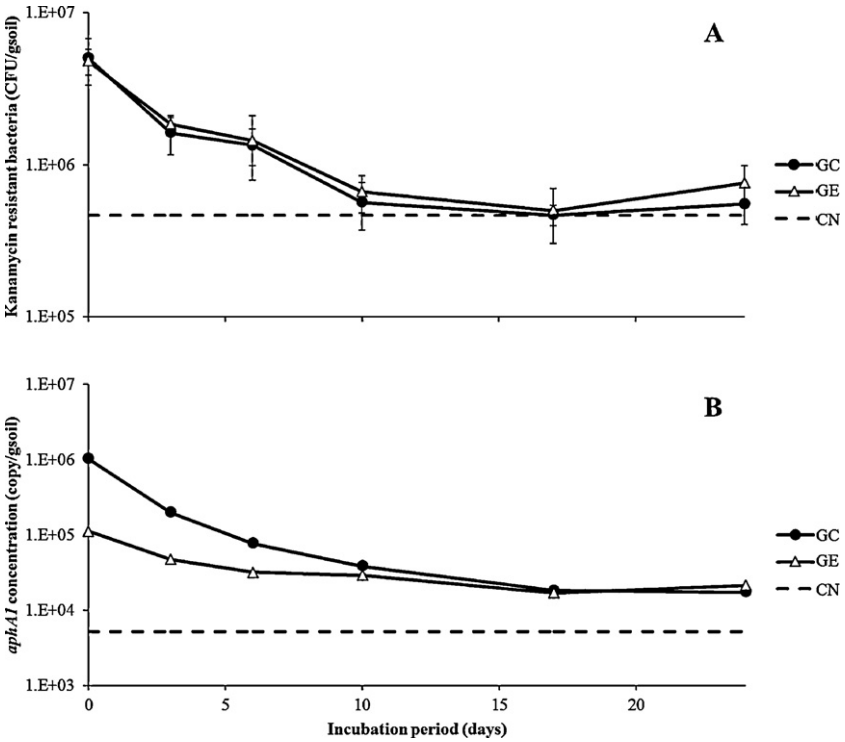

Fig. 1. Temporal variation in (A) kanamycin-resistant bacterial population and (B) *aphA1* gene copies in soil inoculated with GM *C. glutamicum* (GC) and GM *E. coli* (GE). The *Y*-axis error bar indicates the standard error of three independent experiments, and the dashed lines mark average values of the negative control (CN). Data beyond 24 day time points are excluded, since only minor fluctuations were observed.

material (Table S1). The PCR products were purified twice using a QIAquick Gel Extraction Kit and a QIA PCR Purification Kit (Qiagen). The DNA concentrations in the purified PCR products were measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), then pooled in equimolar ratios. The pooled products were further purified using a MinElute PCR Purification Kit (Qiagen). Pyrosequencing was performed using GS FLX Titanium kits (Roche, Branford, CT, USA) and was conducted by Macrogen (Seoul, South Korea).

2.5.2. Analysis of pyrosequencing data

The influence of GM bacteria on the structure of the soil bacterial community was evaluated by analyzing changes in the 16S rRNA gene profile and composition. For this, DNA sequences obtained from pyrosequencing were processed using the pyrosequencing pipeline from the Ribosomal Database Project [13]. The obtained sequences were separated according to the barcodes, and quality filtering was performed (quality score > 20; sequence length > 300 bps; no ambiguous nucleotide) [14]. Chimera Slayer was used to remove likely chimeric PCR products [15]. Sequences were aligned and similar sequences (with >97% similarity) were clustered into operational taxonomic units (OTUs) [16]. The OTUs were used to produce rarefaction curves, and each OTU was classified by taxonomic identity (up to the genus level). Dendrograms were produced from hierarchical clustering results using a Jaccard and Sorenson index calculator. To investigate phylogenetic dissimilarities in bacterial genus, principle coordinate analysis (PCA) was performed using R package vegan. For pathogen analysis, 16S rRNA gene sequences for recognized pathogen genera were isolated by cross checking the taxonomic classification of OTUs with lists of hazardous bacteria obtained from the National Microbial Pathogen Data Resource [17], the Korean government [18], and a study by Bibby et al. The isolated sequences were processed using EzTaxon [19] to identify their similarities with those of recognized pathogens (Table S2).

The nucleotide sequences obtained in this study were deposited in the National Center for Biotechnology Information Sequence Read Archive under accession number SRA048768.

3. Results

3.1. Survival of GM bacteria and persistence of the GM plasmid

In CN, there were $4.6 \times 10^5 \pm 4.4 \times 10^4$ CFU/g soil of kr bacteria (Fig. 1A) and $5.3 \times 10^3 \pm 8.7 \times 10$ copy/g soil of *aphA1* gene copies (Fig. 1B), and these levels were consistent throughout the experiment. The kr bacterial population immediately increased in response to inoculation with GM *C. glutamicum*. However, the population decreased logarithmically in a time-dependent manner and returned to the CN level in 10 days. A similar trend was observed for *aphA1* gene copies (Fig. 1B); however, their decrease was slower than that of the kr bacterial population and stabilized at a concentration approximately 3.5 times greater ($1.9 \times 10^4 \pm 1.6 \times 10^3$ copy/g soil) than the CN level. These results were consistent with those obtained from soil inoculated with GM *E. coli*.

3.2. Shifts in soil microbial ecology

3.2.1. Bacterial diversity and OTU richness

The pyrosequencing yielded a total of 247,225 quality sequences with an average length of 437 bp (range: 377–471 bp). Of the yielded sequences, $88.11 \pm 1.74\%$ were classifiable and were distributed over 35 taxonomic classes (Table S3). The OTU richnesses of GC and WC were equivalent to that CN, but the OTU richnesses of GE and WE were slightly lower than that of CN (Fig. 2). The rarefaction curves for kanamycin-selected soil samples inoculated with *C. glutamicum* (WC10-kr), *E. coli* (WE10-kr), GM *C. glutamicum* (GC10-kr), or GM *E. coli* (GE10-kr) reached plateaus at much lower OTU richness values. Of the yielded sequences, $96.48 \pm 6.65\%$

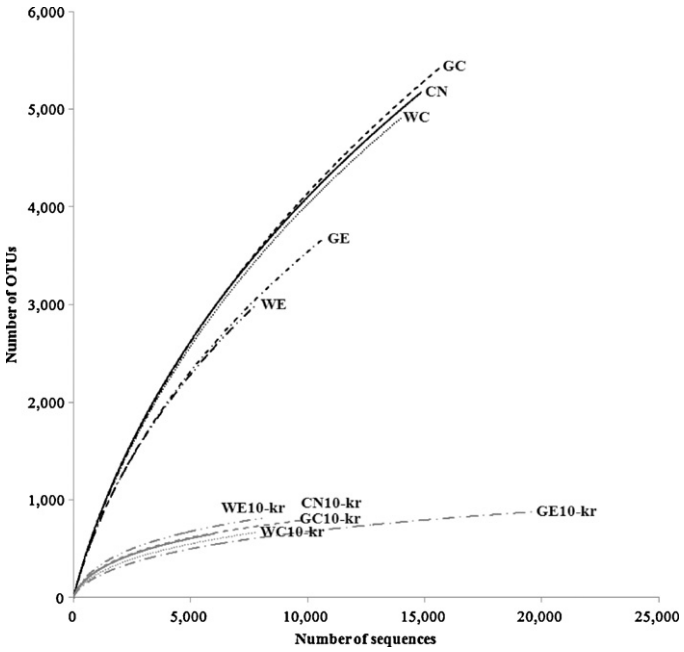

Fig. 2. Rarefaction curves for representative soil inoculated with *C. glutamicum* (WC), *E. coli* (WE), and their GM derivatives (GC and GE) along with negative control (CN). The gray lines represent rarefaction curves for samples selected using kanamycin (marked by 10-kr).

were classifiable and were distributed over 12 taxonomic classes (Table S4). These results indicate a significant reduction in bacterial diversity and OTU richness as a result of antibiotic stress regardless of the inoculum bacteria.

3.2.2. Structure of the bacterial community

In the dendrogram derived using the 16S rRNA gene profile (Fig. 3), the soil microcosms were grouped into two major clusters, with five samples selected using kanamycin in a single cluster and the 13 time-variant soil microcosms in a separate cluster. The latter cluster was further divided into two sub-clusters; one cluster comprised of nine soil microcosms incubated for a short period (0–10 days) and another comprised of four soil microcosms incubated for a long period (38–94 days). The structures of the bacterial communities in the soils inoculated with the tested bacteria showed no significant difference ($p < 0.05$) with those in CN. However, the structure of bacterial communities differed largely between clusters and subclusters (Fig. S1). Such trends were elaborated by the dispersion in the PCA plot (Fig. S2), where the position of GC started to shift after 3 days incubation but keeping close distance to CN and the other microcosms (WC, GE, and WE) at the same incubation times.

In CN, the proportion of the *Corynebacterium* genus was $0.011 \pm 0.003\%$ and was consistent throughout the experiment (Fig. S3). After inoculation with GM *C. glutamicum*, the proportion of *Corynebacterium* exhibited fluctuations analogous to those in the kr bacteria population (Fig. 1A) and the *aphA1* gene (Fig. 1B). Thus, the majority of inoculum bacteria in the soil microcosms seemed to perish within 10 days. At this time point, the proportion of *Corynebacterium* genus in GC was roughly 5% less than in WC. The difference was more evident in the reference experiments, in which GE had roughly 20% less *Escherichia* genus than in WE. Moreover, according to pyrosequencing results, inoculation with test GM bacteria stimulated the growth of a few indigenous soil bacteria. Among them, eight genera (*Gemmatimonas*, *Flavobacterium*, *Steroidobacter*, *Sphingomonas*, *Terrimonas*, *Pseudomonas*, and *Chitinophaga*) showed significant increases in proportion from CN levels.

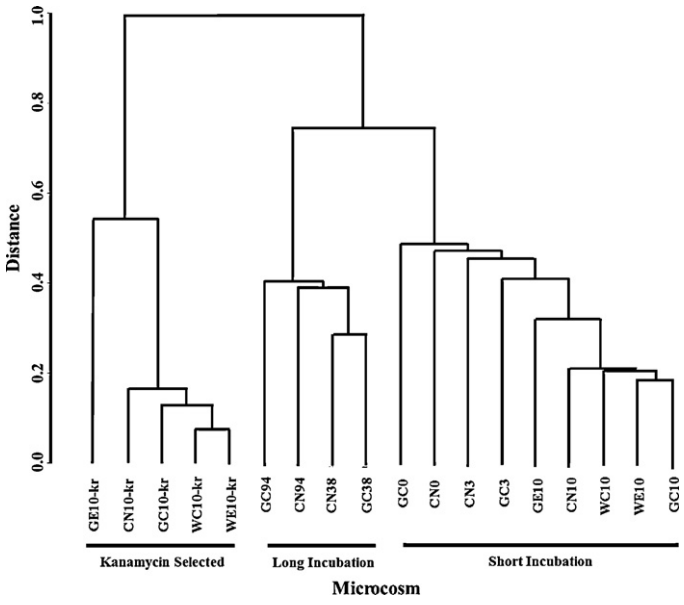

Fig. 3. Dendrogram based on the Jaccard index of 16S rRNA gene sequences obtained from soil inoculated with *C. glutamicum* (WC) and *E. coli* (WE) and their GM derivatives (GC and GE) along with negative control (CN) after (i) short incubation (0–10 days), (ii) long incubation (34 and 94 days), and (iii) kanamycin selection (samples from the 10-day time point, screened with 50 μg/mL of kanamycin). The numbers and kr after the abbreviation indicates incubation time in days and kanamycin selection, respectively.

3.2.3. Structure of the kr resistant bacterial community

After kanamycin selection, the bacterial community showed a clear reduction in diversity (Fig. 2). Despite the change, the bacterial community of GC10-kr demonstrated high homology ($p < 0.05$) with both CN10-kr and the samples derived from soils inoculated with wild-type strains (Fig. 3 and Fig. S1). However, in the PCA analysis, GC10-kr was positioned away from the GE10-kr that possessed a unique bacterial community (Fig. S4). In particular, inoculating *C. glutamicum* and its GM derivative caused dispersion along the PC2 axis, whereas inoculating *E. coli* and its GM derivative showed dispersion along the PC1 axis. Unlike in GC10-kr, excessive growth in the proportion of the *Escherichia* genus was observed in GE10-kr, along with the emergence of the genera *Enterobacter* ($5.122 \pm 0.163\%$) and *Salmonella* ($0.902 \pm 0.059\%$), in significant proportions. This trend was not observed in CN10-kr or in the other tested samples.

3.2.4. Sequences related to pathogens

On day 10, 16S rRNA gene sequences related to pathogens (sequence similarity > 97%) were detected in GC (*Enterococcus faecium*), GE (*Acinetobacter baumannii*), and even CN (*Klebsiella pneumoniae*) (Table 1). The proportion of sequences related to pathogens greatly increased after kanamycin selection. In CN10-kr, sequences related to *Pseudomonas aeruginosa* ($5.620 \pm 1.057\%$), *Bacillus cereus* ($0.112 \pm 0.059\%$), and *Bacillus anthracis* ($0.010 \pm 0.006\%$) were observed. These sequences were present in all other kr bacterial communities as well. In addition to these three pathogens, sequences related to *E. faecium* were observed in GC10-kr ($2.525 \pm 0.625\%$), while *Shigella sonnei* sequences were observed in both WC10-kr ($0.021 \pm 0.014\%$) and WE10-kr ($0.020 \pm 0.006\%$). For GE10-kr, the sequences observed were related to the pathogens *S. sonnei* ($19.334 \pm 0.433\%$), *Shigella boydii* ($2.418 \pm 0.150\%$), *Shigella flexneri* ($0.778 \pm 0.055\%$), and *E. coli*

Table 1
Proportion (%) of 16S rRNA genes showing high sequence similarity (similarity, >97%) with acknowledged pathogens in soil microcosms and kanamycin selected samples at 10 days.

Samples	GC10	WC10	GE10	WE10	CN10
Soil microcosms					
Acinetobacter baumannii	0.004 ± 0.003	–	–	–	–
Enterococcus faecium	0.018 ± 0.009	–	0.012 ± 0.007	–	–
Klebsiella pneumoniae	–	–	–	–	0.008 ± 0.004
After kr selection[a]	GC10-kr	WC10-kr	GE10-kr	WE10-kr	CN10-kr
Bacillus anthracis	0.625 ± 0.054	0.050 ± 0.032	0.003 ± 0.002	0.132 ± 0.001	0.010 ± 0.006
Bacillus cereus	4.506 ± 0.603	0.660 ± 0.421	0.041 ±0.023	2.055 ± 0.457	0.112 ± 0.059
Enterococcus faecium	2.525 ± 0.625	–	–	–	–
Escherichia coli O117:H7	–	–	0.236 ± 0.015	-	–
Pseudomonas aeruginosa	0.089 ± 0.134	9.989 ± 4.424	0.278 ± 0.013	1.640 ± 1.240	5.620 ± 1.057
Shigella boydii	–	–	2.418 ± 0.150	–	–
Shigella flexneri	–	–	0.778 ± 0.055	–	–
Shigella sonnei	–	0.021 ± 0.014	19.334 ± 0.433	0.020 ± 0.006	–

[a] kr selection: kanamycin selected soil sample.

O117:H7 (0.236 ± 0.015%) in addition to the three pathogens previously identified in CN10-kr.

4. Discussion

In this study, releasing commercial GM *C. glutamicum* with kanamycin resistance conferred by the insertion of a GM plasmid containing the *aphA1* gene caused no permanent change in the indigenous kr bacterial population. This was illustrated by the quick restoration in kr bacterial population of the soil inoculated with GM *C. glutamicum* (Fig. 1A). The high kr bacterial population observed at the earlier phase of the experiment was likely due to the direct detection of GM *C. glutamicum*. This was evidenced by the analogous reduction trends for the kr bacterial population and indicators for GM *C. glutamicum*, such as *aphA1* copies (Fig. 1B), and *Corynebacterium* proportion (Fig. S3). These results were consistent with observations from GM *E. coli*. The bacterial populations of soil are known to be regulated by naturally occurring biotic (predation, competition, antagonism, etc.) and abiotic (temperature, pH, moisture, adsorption, etc.) factors [20], and inoculum bacteria must avoid these inhibitors to persist. The potential risk of bacterial inocula increases with their survival rate, as the soil ecosystem is more likely to change with extended bioactivity. GM bacteria may acquire the necessary features to overcome survival barriers through carefully designed GM elements. However, viable cell counts suggested that neither pCJ1-TNAI_L469P nor pCR2.1-TOPO plasmids are equipped with such features. Moreover, pyrosequencing results displayed evidence for reduced survival for GM bacteria compared to their wild-type strains (Section 3.2.2). This may be due to the energy burden required for maintaining the GM plasmid [21,22]. Alternatively, GM plasmids themselves showed longer persistences than their host cells because they are protected by soil particles from DNA-degrading enzymes (Fig. 1B) [23]. GM plasmids are a potential microbial risk, causing the proliferation of antibiotic-resistant bacteria in soil, as there may be indigenous bacteria capable of developing genetic competences and expressing traits acquired from naked DNA [24].

Although GM bacteria quickly perished in soil without altering the kr bacterial population, the biological effects (bio-stimulation, enzyme production, etc.) of their presence could have influenced the dynamics of bacterial communities and ecosystems. As suspected, nutrients released from the dying cells, together with an increase in the water content, seemed to stimulate the growth of some indigenous bacteria (see Section 3.2.2) [25]. Nevertheless, the biostimulation was short-lived and insufficient to cause a bacterial community shift (Fig. 3). However, the 16S rRNA gene profiles derived from pyrosequencing results revealed a general bacterial community shift following an extended incubation period for all tested microcosms. As a majority of the viable inoculum bacteria perished prior to the sampling time, the shift was likely induced by experimental artifacts [26]. This was further supported by the grouping of microcosms of the same incubation times in the PCA plot (Fig. S2), suggesting an insignificant influence of bacterial inocula on the bacterial communities. In previous studies, closed batch microcosms similar to the ones used in this study were typically used for microbial risk assessments. However, unless aging effects can be avoided, comparative assessments with reference microcosms, like the ones used in this study, may be more suitable. The commonly addressed risk criteria reveal an insignificant survival of GM bacteria and insignificant changes to the structure of the bacterial community. Thus, the microbial risk of accidentally releasing GM *C. glutamicum* may be similar to that of releasing wild-type strains. This may apply to other GM bacteria, unless their GM elements are specifically designed to enhance the survival of host bacteria in the environment. However, potential risks associated with natural transformations still linger, as GM plasmids persisted throughout the durations of the experiments.

Although the risk levels for both GM bacteria were acceptable according to the commonly assessed risk criteria, the pyrosequencing analysis of kr bacteria suggested otherwise. Inoculation with GM bacteria seems to have pre-stimulated some indigenous kr bacteria in the soil, causing them to react more vigorously during the kanamycin selection process. The exact nature of the shift is unclear, but the bacteria affected and the severity of the effect seems to vary according to the inoculum strain and its persistence in the soil (Fig. S4). These changes, however, were insufficient to cause a noticeable shift in culturable kr bacterial communities, as indicated by the fact that the GC bacterial community remained similar to that of CN, even after the kanamycin selection process (Fig. 3). Nevertheless, the influence of GM bacteria on kr bacterial communities exhibited a strong dependence on the survival of bacterial inocula, like the kr bacterial population, as massive growth of *Escherichia* during the kanamycin selection process led to the development of a unique kr bacterial community in GE10-kr (Fig. S1). The conflicting results observed between GC10-kr and GE10-kr suggested different stabilities of GM plasmids in the host bacteria. To develop such a high proportion of *Escherichia* in GE10-kr, remnants of GM *E. coli* in GE must flourish during the kanamycin selection process; this implies the high stability of pCR2.1-TOPO plasmid in *E. coli*, even under environmental conditions. Because a contradicting result was obtained in GC10-kr, it is plausible to assume that GM *C. glutamicum* may have either completely perished or lost its kanamycin resistance [27]. For this experiment, the latter explanation is more probable, as similar results have been observed in serial cultivation experiments (data not shown) and *C. glutamicum* is native to soil environments [28]. The high stability of the GM

plasmid seems to promote the emergence of kr bacteria by increasing the possibility of horizontal gene transfer (see Section 3.2.3). Such phenomena are known to be unlikely, unless the host bacteria or GM elements contain the genetic code that promotes gene transfer. However, considering the frequency of naturally occurring transfer events, it is impossible to assume that the transfer of GM elements will not occur in the event of a GM bacterial release [29]. In support of this argument, the transfer of GM elements has been observed in the soil as early as 24 h after inoculation with GM bacteria [30].

The potential microbial risk of GM bacteria is more apparent in the pathogen analysis. Inoculating GM bacteria had an insignificant effect on the abundance of potential soil borne pathogens, as it did on the soil bacterial community. However, a sizable proportion of 16S rRNA sequences obtained after kanamycin selection displayed high affinities toward recognized pathogens such as *E. faecium*, *S. sonnei*, and others (Table 1). This suggests the possible transfer of the GM element or biostimulation by GM bacteria. In addition, many of these strains were undetected in the control. Thus, the frequency of gene transfer may be higher than previously considered. Among tested samples, GE10-kr possessed the highest pathogen diversity. This once again supports the relationship of GM plasmid stability and kr bacteria emergence. Thus, the potential environmental impact of GM bacteria seems to be strongly influenced by the stability of GM plasmids. As anticipated, the effects of GM *C. glutamicum* on soil kr bacteria and pathogens were similar to those of wild-type strains, making it more environmental friendly than GM *E. coli*.

Conventionally Cloning & Sanger sequencing, Denatured Gradient Gel Electrophoresis (DGGE), and Terminal Restriction Fragment Length Polymorphism (T-RFLP) were used to profile bacterial communities in the environment. Nevertheless, their detection sensitivities and population identification specificities in microbial risk assessment as well as acceptable levels of environmental risk, are under debate [11]. Cloning & Sanger sequencing is highly robust and specific in terms of sequencing detail. There have been many attempts to estimate soil bacterial communities using this method [31–33], but none of the estimates provided sufficient detail due to limitations in throughput and cost [34]. Alternatively, DGGE provided moderate resolution data at a higher throughput with less effort. With this method, individual bands can be excised, sequenced, and taxonomically analyzed to a reasonable specificity. However, the method has low resolving power and is prone to errors during gel separation processes [35]. In addition, DGGE is inadequate in evaluating shifts in minor bacteria, as it is only able to separate sequences that constitute >0.5% of amplicon [36]. T-RFLP can yield complex but interpretable profiles [37]. In addition, the results of T-RFLP are more reproducible than those of DGGE and there are comprehensible databases for sequence identification. However, there is a chance of identical TRFs arising from unrelated phyla [38], and sequences must constitute >1% of amplicon to be distinguished from background noise [39]. From a technical perspective, pyrosequencing provides an improved detection of the potential environmental risks of GM bacteria compared to conventional techniques. Pyrosequencing can simultaneously analyze 10,000–100,000 sequences, and these numbers may rise with sequencing efforts. Thus, it detects a broader spectrum of bacteria, including the rare taxa constituting less than 0.01% of amplicon. Therefore, it may be more suited for detecting changes in characteristics of indigenous bacteria [40]. Although pyrosequencing is prone to sequencing errors at homopolymer regions in amplicon sequence [14], software has been developed to remove sequence errors, improving its reliability [41,42].

Pyrosequencing may provide an adequate overview of the bacterial community. However, it is impractical and cumbersome to analyze total environmental bacteria. By applying kanamycin selection, a majority of bacteria unaffected by GM bacteria were effectively discriminated from the microcosms. Although this restricts analysis to culturable bacteria, the spectrum analyzed was sufficient to identify evidence for changes in kr bacteria and GM element transfer, proving the applicability of pyrosequencing in evaluating the spread of antibiotic resistance in the environment. Observations from pyrosequencing may be valuable in evaluating the potential health risks of GM bacteria and provide a focal point for further investigation.

5. Conclusion

It was possible to detect minor changes in the individual constituents of complex bacterial communities by using 16S rRNA gene-targeted pyrosequencing. This allowed a broader analysis of the potential microbial risk of releasing GM bacteria and provided valuable information with respect to the taxon that was strongly influenced. According to this analysis, releasing GM *C. glutamicum* raises an insignificant microbial risk, whereas the reference GM *E. coli* triggered potential hazards associated with the emergence of kr bacteria and pathogens. The stability of GM plasmids in host bacteria seems to influence the potential microbial risk from GM bacteria. Therefore, by carefully designing these GM elements, the environmental impact of GM bacteria may be mitigated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2012.05.031.

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