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Article

Hyperthermophilic composting accelerates the removal of antibiotic resistance genes and mobile genetic elements in sewage sludge

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**Hyperthermophilic composting accelerates the removal of antibiotic resistance
genes and mobile genetic elements in sewage sludge**

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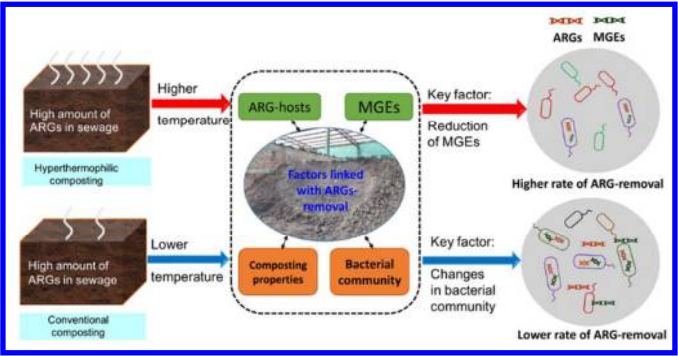
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Abstract

Composting is an efficient way to convert organic wastes into fertilizers. However, waste materials often contain high amount of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) that can reduce the efficacy of antibiotic treatments when transmitted to humans. Because conventional composting often fails to remove these compounds, we evaluated if hyperthermophilic composting with elevated temperature is more efficient at removing ARGs and MGEs, and explored the underlying mechanisms of ARG-removal between two composting methods. We found that hyperthermophilic composting removed ARGs and MGEs more efficiently than conventional composting (89% and 49%, respectively). Furthermore, half-lives of ARGs and MGEs were lower in hyperthermophilic compared to conventional composting (67% and 58%, respectively). More efficient removal of ARGs and MGEs was associated with higher reduction in bacterial abundances and diversity of potential ARG hosts. Partial least squares path modeling suggested that reduction of MGEs played a key role in ARG-removal in hyperthermophilic composting, while ARG reduction was mainly driven by changes in bacterial community composition under conventional composting. Together these results suggest that hyperthermophilic composting can significantly enhance the removal of ARGs and MGEs and that the mechanisms of ARG and MGE removal can depend on composting temperature.

Keywords: Composting, biosolids, temperature, bacterial communities, ARGs

61 **TOC art** (approx. 8.47 cm by 4.76 cm)



Introduction

There is an urgent need to reduce the overuse of chemical fertilizers for economic and environmental reasons^{1,2}. The use of manure-based organic fertilizers are a promising alternative to chemical fertilizers and at the same time provide an efficient mean to process organic wastes. However, therein lies a potential risk: waste products often contain high amount of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs)³ that can reduce the efficacy of antibiotic therapies when transmitted to humans⁴. This is because ARGs often make pathogenic bacteria resistant to clinically used antibiotics⁵ and mobile genetic elements (MGEs), such as plasmids and transposons, can mobilize ARGs between different environment via horizontal gene transfer between different bacteria⁶. Current research suggests that antibiotic resistance genes have become more common in the environment due to heavy use of antibiotics in livestock industries³ and enrichment of ARGs in aquatic environments⁷⁻⁹. For example, wastewater treatment plants (WWTPs), and specifically activated sludge, are important reservoirs for ARGs^{10, 11} where high bacterial abundances and diversity is expected to further promote the horizontal gene transfer of ARGs¹¹. Direct land application of sludge waste as soil amendment (organic fertilizer) is likely to increase the probability of introducing ARGs into soil bacterial communities^{12, 13} from which they could be transferred to vegetables and humans¹⁴. Introducing ARGs to soil could also elevate the risk of transferring ARGs between non-pathogenic and human pathogenic bacteria via horizontal gene transfer^{15, 16}. As a result, correct treatment of sewage sludge is very important to reduce the potential risks of spreading ARGs across agricultural environments.

Various solid waste management practices have been developed for reducing the abundance of ARGs^{17, 18}. For example, bio-drying aeration strategies have been shown to significantly decrease the tetracycline resistance and class 1 integron integrase (*intI*) genes in the sludge¹⁹. Similarly, the addition of zero-valent iron to anaerobic co-digestion of sludge and kitchen waste has also been demonstrated to lead to reduction in the amount of ARGs²⁰. Moreover, high temperatures (55 °C vs. 35 °C) has been shown to be important in reducing ARGs more efficiently from anaerobic

116 digestion sludge¹⁷. Yet, increasing evidence suggests that conventional aerobic
117 composting and anaerobic digestion do not effectively control the proliferation and
118 diffusion of ARGs and MGEs²¹⁻²⁴. Furthermore, reduction of ARGs is often observed
119 only on the short-term and ARGs typically rebound after completion of the
120 treatment^{19, 21}. One potential explanation for this is that ARGs can be located on
121 mobile genetic elements, which can promote their transfer between different bacterial
122 strains and potential ARGs hosts²⁵. Another possible explanation is that thermophilic
123 composting temperature (approximately 55-70 °C) is not high enough for the
124 degradation of the DNA that contain ARGs and/or MGEs even though some of the
125 potential hosts are killed²¹. These few examples suggest that composting is a complex
126 process and that we are still lacking a mechanistic understanding of ARG-removal²¹,
127 ²⁶. For example, it is not clear if the ARG-removal is driven by (1) changes in
128 abundances or community composition of bacteria, (2) physicochemical properties of
129 the compost or (3) both of them^{23, 26, 27}. As a result, a better understanding of the
130 elevated temperature for bacterial communities and gene abundances during the
131 composting is vital for developing more efficient techniques for the removal of ARGs
132 and MGEs^{21, 25}.

133 Here we evaluated the performance of hyperthermophilic composting for the
134 removal of ARGs and MGEs from activated sewage sludge. The hyperthermophilic
135 aerobic composting technique was first developed by Oshima²⁸. During the
136 fermentation process, composting temperatures reach extremely high temperatures of
137 up to 90 °C without exogenous heating, which is 20-30 °C higher compared to
138 conventional composting²⁸. Hyperthermophilic composting has also some other
139 prominent features, such as high bioconversion efficiency²⁹, and has been shown to be
140 associated with distinct microbial communities³⁰. However, there are no published
141 studies on the impact of hyperthermophilic composting on ARGs abundances, and as
142 a result, it is unclear if hyperthermophilic composting is efficient at removing both
143 ARGs and MGEs compared to conventional composting. Here we studied this
144 experimentally by directly comparing these two composting methods. Furthermore,
145 we tried to achieve a more mechanistic understanding of how ARGs are sustained in

the environment by temporally sampling their potential bacterial hosts and looking changes in the entire bacterial community by applying quantitative PCR (qPCR) and Illumina sequencing of bacterial 16S rRNA genes. We hypothesized that: (i) hyperthermophilic composting is more efficient at removing both ARGs and MGEs than conventional composting; (ii) the two composting methodologies will select distinct bacterial communities during the composting; (iii) higher efficiency of ARG-removal is associated with a reduced frequency of potential ARG hosts; and/or, (iv) limits the changes of horizontal gene transfer by more efficiently removing MGEs.

Materials and methods

Conventional and hyperthermophilic aerobic composting setup

Here we compared how two composting processes, conventional and hyperthermophilic aerobic composting, affect the abundance of ARGs, MGEs and the diversity and composition of bacterial communities. Our experiments were carried out in a full-scale sludge hyperthermophilic aerobic composting plant located in Shunyi district, Beijing, China. The detailed process of hyperthermophilic aerobic composting technology has been described previously by Liao *et al.*³¹. Briefly, raw materials including dewatered sewage sludge (with around 75% moisture content; Shunyi WWTPs, Beijing, China) and composting end-products (with around 40% moisture content including 5% rice husk) from the previous composting round were first thoroughly mixed with a ratio of 1:3 (v/v) to adjust the initial moisture content to approximately 60% (with C:N ratio around 8). The compost mixture (approximately 200 tons) was then loaded to the fermentation compartment (8.5 m length, 6 m width and 3.2 m height) up to 2.5 m in height. Forced aeration via two PVC tubes running underground from bottom to the top of the composting pile were supplied according to aeration needs of hyperthermophilic composting³¹. To mix the compost substrate well and to reduce pile-edge effects, mechanical turning of composting material was performed at every four days using pile-specific forklifts to prevent cross-contamination between the piles. Conventional composting followed a

176 previously described protocol by Tortosa *et al.*³². Briefly, the same raw materials were
177 used for conventional and hyperthermophilic composting to build a trapezoidal pile of
178 about 20 tons. Fresh air was supplied naturally without forced aeration by turning the
179 composting material at every two days during the composting process.
180 Hyperthermophilic composting takes normally 25 days according to the experience of
181 the compost factory (Liao; personal communication). In contrast, conventional
182 composting takes around 45 days. Hence, both composting treatments were run
183 synchronously for 45 days but the time after 25 days in hyperthermophilic composting
184 treatment was regarded as storage stage in this study. In both treatments, the main
185 composting compartment or pile was diagonally split into 5 independent replicate
186 piles (N=5). Based on the experience of the compost factory, five thermometers were
187 placed in 40-50 cm depth for daily monitoring of the maximum fermentation
188 temperatures.

189

190 **The sample collection and DNA extraction**

191 We collected samples from both composting treatments at days 0, 2, 4, 7, 9, 15, 21, 27,
192 33, and 45 as follows. To obtain well-distributed and homogenized samples, five
193 subsamples per replicate segment were collected in 40-50 cm depth, mixed together
194 (5000 g) and divided into two aliquots of which one was stored in liquid nitrogen for
195 biological analyses and the other stored at 4 °C for physicochemical analyses. This
196 sampling approach was chosen to reduce the potential bias caused by heterogeneity of
197 the original composting substrate. The total genomic DNA was isolated using the
198 MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) by
199 following manufacturer's protocol. The DNA extraction was conducted three times
200 for each sample and the DNA extracts were combined before the sequencing. The
201 DNA content and the quality was checked with NanoDrop ND-2000 (Thermo Fisher
202 Scientific, Wilmington, USA) and on 1% agarose gel.

203

204 **Determination of physicochemical soil properties during composting**

205 Following physicochemical properties were measured during the composting process

206 using methods described previously^{32, 33}: pH, electrical conductivity (EC), water
207 content (WC), total nitrogen content (TN), total carbon content (TC), total organic
208 carbon content (TOC), total sulfur content (TS), inorganic carbon content (IC),
209 electrical conductivity (EC) and ammonium (NH_4^+), and nitrate (NO_3^-) concentrations.
210 Samples were oven-dried at 105 °C for 24 h to determine moisture content. EC and
211 pH were determined using a conductivity meter (Radiometer, model CDM210) and a
212 pH meter (PB-10, Sartorius, Germany), respectively. NH_4^+ and NO_3^- were measured
213 by a continuous-flow autoanalyser (FlowSys, Systea, Rome, Italy). TOC and IC were
214 quantified using an automatic TOC analyzer for liquid samples (Shimadzu TOC-L
215 CPH, Kyoto, Japan). The TN, TC, and TS were determined with Elementar
216 instrument (Vario MAX cube, Hanau, Germany) using dry combustion and the TN
217 and TC values were used to calculate the C/N ratio.

218

219 **Real-time quantitative PCR (qPCR) for determining antibiotic resistance gene** 220 **and mobile genetic element abundances**

221 Because tetracycline, macrolide, sulfonamide, and aminoglycoside resistance genes
222 are the most abundant ARGs in the sewage sludge²³, we specifically chose to focus on
223 these genes in this study (including ten tetracycline resistance genes (*tetA*, *tetB*, *tetC*,
224 *tetG*, *tetL*, *tetM*, *tetQ*, *tetO*, *tetW*, and *tetX*), six macrolide resistance genes (*ermB*,
225 *ermF*, *ermT*, *ermX*, *mefA*, and *ereA*), seven aminoglycoside resistance genes (*aacA4*,
226 *aadA*, *aadB*, *aadE*, *aphA1*, *strA*, and *strB*) and three sulfonamide resistance genes
227 (*sul1*, *sul2*, and *sul3*). We also measured changes in the abundance of five genes
228 linked with mobile genetic elements such as integrases (*int11*, *int12*), plasmids (*ISCR1*,
229 *IncQ*) and transposons (*Tn916/1545*, abbreviated as *Tn916*) and determined changes
230 in bacterial cell densities by amplifying 16S rRNA gene copies using SYBR-Green
231 real-time qPCR. The primers, annealing temperatures, and amplification protocols for
232 all gene targets are listed in the supplementary material (Table S1). The qPCR and
233 plasmid constructions were conducted according to a previous protocol³⁴ using the
234 LightCycler 96 System (Roche, Mannheim, Germany). Briefly, the plasmids carrying
235 target genes were obtained from TA clones and extracted using a TIAN pure Mini

236 Plasmid kit (Tiangen, Beijing, China). The standard plasmid concentrations (ng/mL)
237 were determined with the Nanodrop ND-2000 (Thermo Fisher Scientific, Wilmington,
238 USA) to calculate gene copy concentrations (copies/mL). The qPCR was carried out
239 in 96-well plates containing 10 μ L of GoTaq qPCR Master Mix (Promega, Madison,
240 USA), 1.5 μ L each of forward and reverse primers (4 mmol/L), 1 μ L of template
241 genomic DNA and 6 μ L of nuclease-free water. Each qPCR run began with 2 min of
242 initial denaturation at 95 $^{\circ}$ C, followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 30 s,
243 annealing for 30 or 45 s according to the length of target at the primer-specific
244 annealing temperature, and extension for 30 s at 72 $^{\circ}$ C. The amplification efficiencies
245 of different PCR reactions ranged from 90% to 110% with R^2 values higher than 0.99
246 for all standard curves. Each reaction was run in triplicate along with standard curves
247 and a negative control where the template genomic DNA was replaced with DNA-free
248 water. The relative abundances of target genes are presented as gene copy numbers
249 per 16S rRNA gene, while the absolute abundances of target genes are shown as gene
250 copy numbers per gram dry sample.

251

252 **High-throughput sequencing and bioinformatics analyses**

253 To determine changes in bacterial community composition during composting, we
254 amplified the V4-V5 region of the bacterial 16S rRNA gene using 515F/907R primers.
255 The reverse primer contained a unique barcode for each sample and the DNA was
256 amplified in triplicate before sequencing with Illumina Hiseq 2500 platform
257 (Guangdong Magigene Biotechnology Co.Ltd, Guangzhou, China). Trimmomatic
258 software (version 0.33) was used to trim the reads with low base quality. The high
259 quality sequences were processed with QIIME pipeline to determine alpha and beta
260 diversity³⁵. The sequences were clustered into OTUs at 97% level similarity using
261 Uclust clustering³⁶. A set of representative sequences from each OTU were assigned
262 taxonomically using a Ribosome Database Project Classifier with a confidence
263 threshold of 0.80 as described previously³⁷. Rarefaction curves were calculated to
264 compare bacterial OTU diversity between different samples. The alpha diversity of
265 each sample was determined as Chao1, Shannon, Observed species and Simpson

diversity indexes. Rarefaction curves were calculated to compare bacterial OTU diversity between different samples. The beta diversities of each composting treatment were analyzed with principal coordinate analysis (PCoA) based on Bray-Curtis distance matrix.

Correlation between different bacterial taxa and ARGs/MGEs during composting

We used local similarity analysis (LSA) to determine correlations between relative abundance of OTUs or annotated taxa and ARGs/MGEs during composting³⁸⁻⁴⁰. The LSA is an optimized method to detect non-linear, non-random, and time-sensitive relationships based on correlation networks^{39, 40}. To reduce computing time and network complexity, only OTUs and taxa with relative abundance of 0.05% or higher were included in the analysis. Similarly, only highly significant ($P < 0.01$) cases with high local similarity scores were retained for further analysis. Finally, q-value (false-discovery rate, Benjamin Hochberg, $q < 0.01$) was applied to correct the P -values and to control the false-discovery rate for multiple comparisons. The retained LS interactions between ARGs and bacterial taxa were visualized as a network in Cytoscape v3.4.0 and network statistics analyzed with Network Analyzer as undirected networks using default settings⁴¹.

Statistical analysis

A first-order kinetic model (ExpDec1) was used to fit the reduction in the abundance of target genes (gene copies per gram of dry sludge) during composting (Origin 9.0, Microsoft, USA)¹⁹. To analyze correlations between ARGs and bacterial taxa, PCoA (Bray-Curtis distance based), redundancy analysis (RDA), Adonis test, and Procrustes tests were performed in R 3.3.2 with vegan package v2.4-3. Effect Size (LEfSe) Linear Discriminant Analysis (LDA) was used to compare differences between conventional and hyperthermophilic composting at the genus level⁴². Discriminating features were identified using the following parameters: (1) the alpha value of factorial Kruskal-Wallis test between classes was set to 0.01 and (2) the threshold of the logarithmic LDA score was set to 2.0. Partial least squares path modeling

(PLS-PM) was employed to explore the direct, indirect and interactive effects between all measured variables for ARG abundances (The R package plsrm (v 0.4.7))⁴³. PLS-PM is a powerful statistical method to study interactive relationships among observed and latent variables^{43, 44} and is widely applied to explain and predict relationships in multivariate data sets⁴⁴⁻⁴⁶. The model included the following variables: composting temperature, physicochemical composting properties (WC, TC, EC, pH, IC, C/N, TN, TOC, NH_4^+ , NO_3^-), bacterial community composition (based on OTU abundances) and MGE and ARG abundances (relative target gene abundances, i.e., standardized by total bacterial abundances). Indirect effects are defined as multiplied path coefficients between predictor and response variables including all possible paths excluding the direct effect. The final model was chosen of all constructed models based on the Goodness of Fit (GoF) statistic - a measure of the model's overall predictive power.

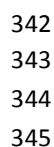
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310 Results

311 Hyperthermophilic composting is more efficient at removing ARGs and MGEs 312 compared to conventional composting

313 The temperature profiles of the two composting treatments were clearly different
314 (Figure S1). The temperature of hyperthermophilic treatment rapidly increased to about
315 90 °C after 24 hours of fermentation, while in the conventional composting, the
316 temperature raised with much slower rate and reached maximum temperatures of 60 °C
317 after 18 days of fermentation. All targeted 25 ARGs and 3 MGEs were detected in all
318 samples; either of the plasmids (*ISCR1* and *IncQ*) was not detected in any of the
319 samples. Mean concentrations of ARGs and MGEs were approximately 5.1×10^{11} and
320 1.1×10^{10} gene copies per gram (dry weight) of initial raw sludge, respectively, with
321 tetracycline and sulfonamide resistance genes being the most dominant ARGs
322 accounting for 64.8%-93.5% of all ARGs (Figure S2). At day 4, hyperthermophilic
323 composting was more efficient at reducing aminoglycoside and macrolide resistance (64%
324 and 84%, respectively) compared to conventional composting (31% and 41%,
325 respectively, $P < 0.01$, Figure 1a). After 21 days of composting, the removal rates of

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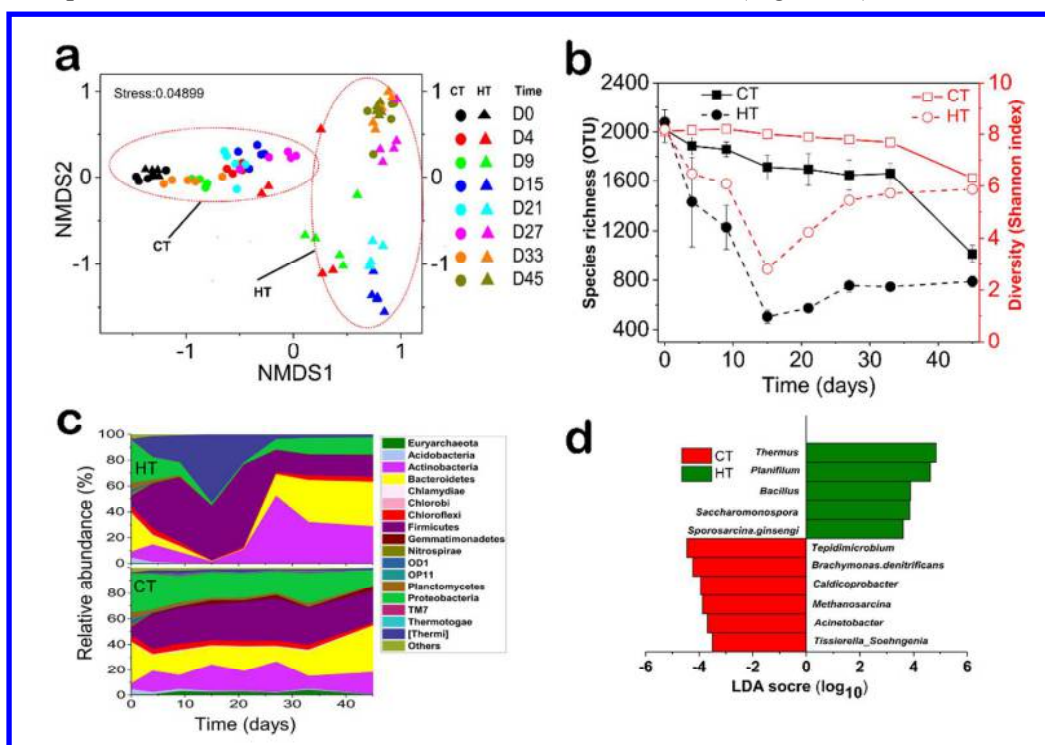
X-axis indicate genes conferring resistance to tetracyclines (Tet), sulfonamides (Sul), aminoglycosides (Amin), macrolides (Mac), and genes encoding mobile genetic element (MGEs). An asterisk (*) and two asterisks (**) indicate significant differences at 0.05 and 0.01 significance levels, respectively. Panel (d): The abundance dynamics of total ARGs (left Y-axis) and MGEs (right y-axis) in two composting treatments. Panel (e): Heat maps showing the mean abundance of normalized ARGs and MGEs (copies per 16S rRNA gene) in both composting treatments. Red and green colors indicate high and low gene abundances, respectively. All target gene abundances are shown as the relative abundances.

354

355 **Hyperthermophilic and conventional composting leads to distinct bacterial** 356 **communities**

The two composting treatments selected for distinct bacterial communities during the 45 days of the experiment (Adonis test, $P < 0.001$), while no difference was observed at the last time point (at day 45; non-metric multidimensional scaling plot (NMDS): Figure 2a and PCoA analysis: Figure S3). We also found that the bacterial community composition (at phylum level) varied more intensively in time under hyperthermophilic composting during the thermophilic phase (day 2 to 15, Figure 2c), while both total bacterial abundances (16S rRNA gene copy numbers) and bacterial community diversity were lower in hyperthermophilic compared to conventional composting especially ($P < 0.01$, Figure 2b, Figure S4). More specifically, hyperthermophilic composting reduced the relative abundance of Proteobacteria and Bacteroidetes from 32.1% to 2.0% and 30.6% to 0.32% by day 15, respectively (Figure 2c). Correspondingly, the abundance of thermophilic phyla, Thermi and Firmicutes (consisting principally of the class Bacilli), increased from 0.41% to 53.1% and from 8.0% to 42.3% by day 15, respectively (Figure 2c). As a result, the abundances of the two most dominant genera, *Thermus* (53.1%) and *Planifilum* (26.7%), belonging to Thermi and Firmicutes, were 86 and 37 times higher in hyperthermophilic compared to conventional composting (Figure 2d). The most dominant genera in the conventional composting were *Tepidimicrobium*, *Brachymonas*, *Actinomadura*, and *Acinetobacter*. These bacterial community structure differences were further confirmed by the linear discriminant analysis (LDA) effect size tool LEfSe (Figure 2d). Notably, Proteobacteria, including classes of

378 Gammaproteobacteria, Betaproteobacteria, and Alphaproteobacteria, were dominant
 379 discriminating key groups in the conventional treatment, whereas Thermi and
 380 Firmicutes, mainly including class Bacilli, were the key discriminating groups in the
 381 hyperthermophilic treatment (Figure S5). Towards the end of the experiment, the
 382 composition of bacterial communities became more similar (Figure 2c).



383
 384 **Figure 2. Changes in bacterial community composition and diversity under**
 385 **hyperthermophilic (HT) and conventional composting (CT).** Panel (a): The overall distribution
 386 pattern of OTU-based bacterial community dissimilarity in the two composting treatments (based
 387 on non-metric multidimensional scaling (NMDS); ordination derived from weighted-UniFrac
 388 distances). Circles denote for conventional and triangles for hyperthermophilic composting and
 389 different colors denote for different sampling days. Panel (b): Changes in bacterial community
 390 species richness (left Y-axis) and alpha diversity (Shannon index; right Y-axis) in the two
 391 composting treatments. Panel (c): The relative abundance of different bacterial phyla in the two
 392 composting treatments. (d): Histogram of the LDA scores for discriminating bacterial genera that
 393 showed clear abundance differences between hyperthermophilic and conventional composting
 394 treatments (genus level, LDA-score > 3.5).

396 **Correlations between ARG, MGE and bacterial taxa abundances**

397 Based on procrustes analysis, gene abundances of ARGs were significantly correlated
 398 with the bacterial community composition in both composting treatments (Figure S6).

399 Similarly, ARGs and MGEs were significantly correlated with each other ($P < 0.001$)
400 in both composting treatments (Figure S7). Local similarity and network analysis to
401 link ARGs, MGEs and bacterial taxa abundances revealed that most ARGs and MGEs
402 correlated significantly ($P < 0.01$) with 52 and 31 bacterial taxa (at genus level)
403 within conventional (Table S2) and hyperthermophilic (Table S3) composting
404 treatments, respectively. Of all ARG-associated bacteria, 17 genera were common for
405 both treatments, 14 genera were only detected in the hyperthermophilic and 35 were
406 detected only in the conventional composting treatment (Figure 3a). More than 50%
407 of bacteria that significantly correlated with ARGs and MGEs belonged to
408 Proteobacteria and Bacteroidetes, the two dominant taxa in initial raw sludge samples
409 (Figure S8). Interestingly, the densities of *Acinetobacter*, *Dokdonella*, and *Fusibacter*
410 correlated with both ARG and MGE abundances in both composting treatments, while
411 *Methanobacterium* (archaea) densities correlated with ARGs and MGEs only in the
412 hyperthermophilic composting. ARGs and MGEs were significantly clustered in the
413 networks ($P < 0.01$, Figure S9). For example, the cluster of resistance genes around
414 *intI1* and *intI2*, and *Tn916* ($P < 0.01$) consisted of known gene cassettes associated
415 with MGEs. Together these results suggest that bacterial taxa that correlated
416 positively with ARGs and MGEs could have played an important role for the
417 proliferation of resistance genes during composting.

418 We next focused on comparing the associations between ARGs, MGEs and
419 bacterial taxa in both composting treatments. The majority of ARG-associated
420 bacteria (17.9% of all sequences) in the initial raw sludge belonged to *Acinetobacter*
421 (2.3%), *Bacteroides* (4.0%), *Dechloromonas* (4.5%), *Nitrospira* (3.1%), and
422 *Paludibacter* (3.8%, Table S4-5). The abundance of these taxa decreased more in the
423 hyperthermophilic compared to the conventional treatment during the composting
424 (Figure 3b). A similar trend was also found at the family level: the mean abundance of
425 ARG-associated bacteria belonging to families Moraxellaceae, Bacteroidaceae,
426 Rhodocyclaceae, Nitrospiraceae, and Porphyromonadaceae sharply decreased from
427 46.6% to 15.3% in the hyperthermophilic treatment after 4 days of composting
428 (Figure 3b). The densities of these bacteria remained low (<5%) throughout the

experiment (from day 4 to 45) in the hyperthermophilic composting, while the relative abundances of those taxa were maintained at an elevated level (30%–48.3%) until day 33 in the conventional composting treatment ($P < 0.05$, Figure 3b). Together these results suggest that hyperthermophilic composting reduced the abundance of potential ARG bacterial host taxa more efficiently compared to conventional composting.

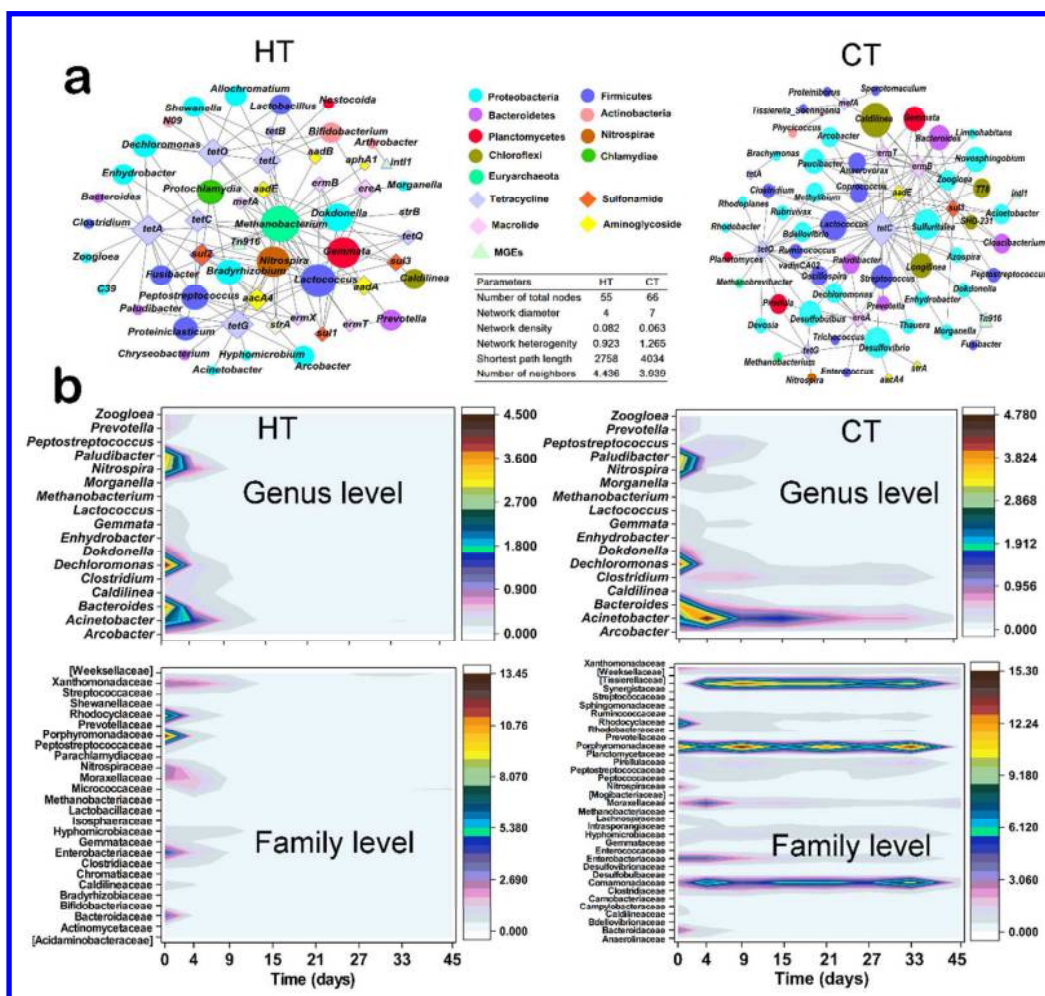


Figure 3. The relationship and the abundance of ARG- and MGE-associated bacteria during hyperthermophilic (HT) and conventional (CT) composting. Panel (a): Co-occurrence network analysis showing the associations between ARGs/MGEs and bacterial taxa in both composting treatments. Panel (b): Distribution profiles showing the relative abundance of ARG- and MGE-associated bacteria at genus (upper panels) and family (lower panels) level in both composting treatments. The legend on the left side denote for taxonomic groups and the legend on the right side the relative bacterial abundances (%) based on total 16S rRNA gene sequences for each presented taxa. The network analysis of all gene abundances are based on the relative abundances.

444

445 **Determining the direct and indirect relationships between composting**
446 **temperature, physicochemical composting properties, bacterial community**
447 **composition and MGE abundance for the abundance of ARGs**

448 The RDA analysis explained 89.7% and 73.0% of the total variance of ARG
449 abundances in hyperthermophilic and conventional composting treatments,
450 respectively (included variables: composting temperature and properties, bacterial
451 community composition and MGE abundances, Figure S10). To explore the effects of
452 composting temperature, composting properties, bacterial community composition
453 and MGEs on the ARG abundances in more detail, we constructed a partial least
454 squares path model (PLS-PM) to assess the direct and indirect effects between
455 observed (indicators) and latent constructs (Figure 4). We found that composting
456 temperature had similar positive or negative direct effects on composting properties,
457 bacterial community composition and ARG and MGE abundances in both composting
458 treatments (Figure 4). However, the link between temperature and MGE abundances
459 was only significant in the hyperthermophilic composting. Composting properties had
460 only significant negative direct effects on the bacterial community composition in
461 both treatments, while the bacterial community composition had significant positive
462 direct effects on the abundances of MGEs and ARGs in both treatments. Crucially,
463 MGE abundances strongly explained the ARG abundances in the hyperthermophilic
464 composting, while the direct effect of bacterial community composition was more
465 important factor in the conventional composting (Figure 4a-b). These results suggest
466 that ARG abundances were affected by different mechanisms in hyperthermophilic
467 and conventional composting treatments.

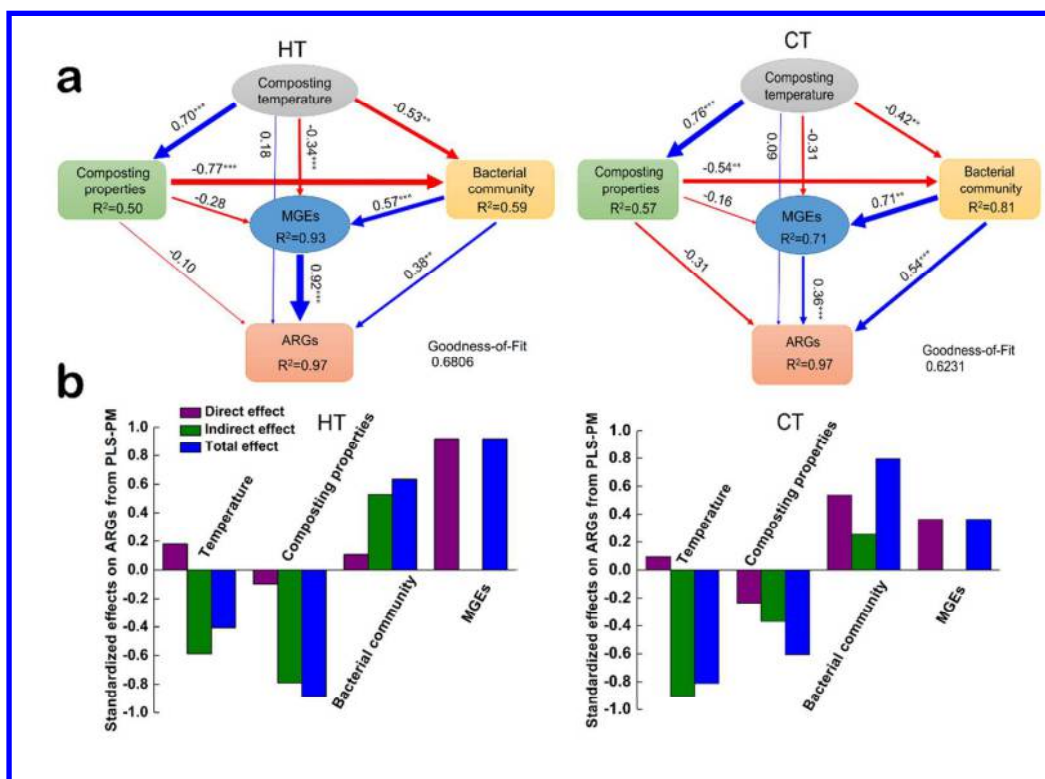


Figure 4. Partial least squares path model (PLS-PM) showing the direct and indirect effects of different factors on ARG abundances in hyperthermophilic (HT) and conventional composting (CT). Panel (a): PLS-PM describing the relationships between temperature, composting properties, bacterial community composition and MGE abundances on ARG abundances in hyperthermophilic and conventional composting. Larger path coefficients are shown as wider arrows and blue and red colors indicate positive and negative effects, respectively. Path coefficients and coefficients of determination (R^2) were calculated after 999 bootstraps and significance levels are indicated by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$). The Goodness of Fit (GoF) for the hyperthermophilic and conventional treatments was 0.68 and 0.62, respectively. Panel (b): Standardized direct and indirect mean effects derived from the partial least squares path models. All target gene abundances are presented as relative abundances.

Discussion

Hyperthermophilic composting is more effective at reducing ARG and MGE abundances compared to conventional composting

Temperature played a crucial role for the rate and level of ARG and MGE removal in our experiment. It is well known that high temperature is the principal factor controlling the inactivation of pathogenic microorganisms in organic waste⁴⁷. Despite large temperature differences, no significant difference in total ARG abundances were

488 observed before day 4 between the two composting treatments. This suggests that
489 degradation of ARGs needs a longer exposure at high temperatures. Relatively long
490 incubation period at over 70 °C temperature in thermophilic composting treatment (15
491 days) might thus have been important factor contributing the high ARG-removal rate.
492 Although the maximum temperature of traditional composting reached up to 60 °C
493 (>55 °C for approximately 5 days), most of the quantified ARGs still persisted and
494 some ARGs even increased in abundance in time (Figure 1c). This persistence of
495 ARGs could be due to the presence of some heat tolerant hosts of ARGs or horizontal
496 transfer of ARGs via MGEs. Laboratory studies have suggested that temperatures
497 above 70 °C are required to completely and directly degrade bacterial DNA⁴⁸, which
498 could explain our observed increased removal of ARGs and MGEs in
499 hyperthermophilic compared with conventional composting. In addition, antibiotic
500 residues in the waste and composting products could have affected the emergence of
501 ARGs^{49, 50}. However, most antibiotics degrade very rapidly ($t_{2/1}$ =0.9 to 9 days) in
502 thermophilic composting according to previous studies^{51, 52}. Crucially, we used the
503 same raw materials for conventional and hyperthermophilic composting, and hence,
504 the effect of potential antibiotic residues unlikely affected the difference in
505 ARG-removal in this study. We also found that the $t_{1/2}$ of all tested target genes was
506 shortened in hyperthermophilic compared to conventional composting, and in the case
507 of genes *intI1*, *Tn916*, *tetB*, and *sull*, the $t_{1/2}$ of most ARGs and MGEs was lower than
508 previously reported^{19, 53, 54}. Together these results suggest that hyperthermophilic
509 composting was more efficient at removing ARGs and MGEs.

510 While the abundances of ARGs remained lower in hyperthermophilic compared to
511 conventional composting, the abundances of ARGs also increased during the ‘storage
512 stage’ of the hyperthermophilic composting (Figure 1d). This could have been caused
513 by regrowth of certain bacterial ARG hosts due to a decrease in the composting
514 temperature (Figure 1d). However, this increase in ARG abundances was not
515 associated with an enrichment of MGEs (Figure 1d), which suggests that this
516 secondary ARGs dissemination was not driven by horizontal gene transfer. From a
517 practical perspective, this result suggests that composting products should not be

518 stored for extended periods of time, in our case of weeks, due to risk of increase in
519 ARGs abundances. In particular, some ARGs such as *tetX*, *tetW*, *sul1*, *sul2*, and *ermF*
520 were still found in reasonably high abundances in the compost, suggesting that they
521 are extremely tolerant to high temperatures, or alternatively, can use thermophilic
522 bacteria as their hosts. This is in line with previous studies showing that some heat
523 tolerant ARGs are not removed during composting^{19, 23}, and hence, some
524 complementary strategies are needed to attain complete removal of all types of ARGs.
525 Among five tested MGEs, genes encoding two integrases (*intI1* and *intI2*) and one
526 transposon (*Tn916*) but not any plasmid genes (*ISCR1*, *IncQ*) were detected in any of
527 the samples. This suggests that horizontal gene transfer of ARGs was mainly driven
528 by integrases and transposons. In the future, higher numbers of MGEs and ARGs
529 should be studied using high-throughput quantitative PCR approaches to build a more
530 complete picture of the role of horizontal gene transfer for the resistome during
531 composting. Our findings suggest that the temperature applied in conventional
532 composting was likely not high enough to degrade ARGs and MGEs directly. Instead,
533 the reduction of ARGs and MGEs was probably caused by decrease in the abundance
534 of ARG and MGE hosting bacteria^{55, 56}. In contrast, periodically extremely high
535 temperatures could have directly broken down ARGs and MGEs during
536 hyperthermophilic composting. This idea is also supported by the PLS-PM results that
537 revealed direct effects of hyperthermophilic composting on ARGs and MGEs (Figure
538 4).

539 **Hyperthermophilic composting alters the bacterial community composition and** 540 **ARG-bacterial taxa associations**

541 NMDS analysis revealed that the bacterial community composition differed between
542 conventional and hyperthermophilic composting until day 33, but no difference was
543 observed at day 45 (the end). This suggest that bacterial communities converged
544 between two composting treatments when the composting treatments reached similar
545 temperatures and physicochemical properties⁵⁷. Compared to conventional
546 composting, hyperthermophilic composting led to reduced total bacterial abundances
547 and lowered species richness and bacterial community diversity (Figure 2b and Figure

S4). These effects could have important indirect effects on ARGs and MGEs. First, the reduction in total bacterial densities could have constrained the horizontal transfer of ARGs via less frequent encounter rates⁵⁸. Second, loss of diversity could have resulted in the reduction of suitable ARG and MGE host bacteria. In line with these hypotheses, we found that bacteria belonging to two phyla (Figure 3), Proteobacteria and Bacteroidetes that are common hosts of ARGs, were dominant in the raw sludge^{59, 60}, but observed at significantly reduced abundances in the hyperthermophilic treatment (Figure S8b). According to previous studies^{59, 61}, the majority of the bacteria (>50%) associated with ARGs and MGEs belonged to Proteobacteria and Bacteroidetes. In contrast, extreme thermophiles belonging to the genera *Thermus* and *Planifilum* dominated (89% relative abundance, Figure 2c) the thermophilic phase of the hyperthermophilic composting. Crucially, both genera are not associated with ARGs or MGEs⁶². Even though hyperthermophilic and conventional composting resulted in a distinct bacterial community composition (Figure 2c), this difference gradually decreased towards the later stages of the composting when the temperature of both treatments fell back to normal. Crucially, even though the abundance of Proteobacteria and Bacteroidetes increased during the later stages of hyperthermophilic composting, the abundance of ARGs increased only slightly, while an obvious increase in ARGs abundances was observed in the conventional composting (Figure 1e). One reason for this is that most of the potentially ARGs-linked bacterial host taxa were killed during the extremely high-temperature composting phase. Alternatively, reduction in the diversity and abundance of horizontal gene transfer agents (MGEs) could have constrained further reinfection of suitable hosts. To study the associations between ARGs and bacterial taxa in more detail, we performed combined bacterial network and LSA analysis, which are powerful tools to indirectly explore potential co-dependencies based on co-occurrence relationships⁴⁰. In agreement with previous studies⁶³⁻⁶⁵, we found that *Bacteroides*, *Clostridium*, *Enterococcus*, and the archaeon *Methanobrevibacter* were positively associated with ARGs. These potential ARG hosts were strongly reduced in the hyperthermophilic treatment, suggesting that these potential ARG hosts were killed

578 during the composting (Figure 3a). This conclusion was further confirmed using the
579 relative abundance data obtained from high-throughput sequencing for each host
580 (Figure 3b). Conversely, the dominant genera in conventional composting were
581 *Brachymonas*, *Acinetobacter*, *Tissierella*, *Soehngenia* that all were positively
582 associated with ARGs or MGEs. Together these results suggest that both density- and
583 diversity-mediated effects improved the removal of ARGs in hyperthermophilic
584 composting by reducing the occurrence of horizontal gene transfer and by directly
585 killing potential ARG-host bacteria.

586

587 **Hyperthermophilic and conventional composting had potentially different** 588 **underlying mechanisms for ARG-removal**

589 To explore complex relationships between composting temperature, composting
590 properties, bacterial community composition and MGE abundances on ARG
591 abundances, we conducted a PLS-PM analysis. We found that ARG abundances were
592 not directly affected by composting temperature. This was contradicting our
593 hypothesis that composting temperature was the main and direct contributor of ARGs
594 reduction. However, it is in line with a previous study showing that the bacterial
595 community rather than the composting temperature was the major direct factor
596 affecting the abundance of ARGs²³. Our model suggests that underlying mechanisms
597 behind the ARG-removal were different for hyperthermophilic and conventional
598 composting. More specifically, MGE abundances had strongest direct influence on
599 ARG abundances in hyperthermophilic composting. In contrast, bacterial community
600 composition was the major determinant of ARG abundances in the conventional
601 composting. However, in both treatments, bacterial community composition and
602 MGE abundances were significantly correlated with composting temperature (Figure
603 4), and most importantly, showed correlations in the same direction even though the
604 magnitude was different. This suggests that both MGEs and the bacterial community
605 composition determined the ARG abundances in both composting treatments but that
606 the relative importance of these factors was different. In hyperthermophilic
607 composting, ARG abundances appeared to be more strongly limited by less frequent

horizontal gene transfer as MGEs were almost completely removed. In contrast, the dynamics and the abundance of potential bacterial hosts played a more important role in conventional composting. Based on our PLS-PM analyses (Figure 4a), MGEs were shown to be direct transfer agents of ARGs and no indirect effects were found. However, other factors including composting temperature, composting properties, and bacterial community composition had a profound effect on ARGs which were partly direct (e.g. in hyperthermophilic composting) or indirect via changes in the bacterial community composition (conventional composting). Most ARG cassettes are found in MGEs such as integrons located on transposons and broad-host range plasmids⁶⁶. We also found that most bacterial taxa were associated with more than one ARG subtype (Figure 3b) and that ARGs and MGEs were highly correlated in both treatments (Figure S7). This further supports the idea that ARGs were carried on MGEs that could have mobilized ARGs between different bacterial taxa.

In conclusion, this study demonstrates that hyperthermophilic composting is an efficient and powerful methodology for decreasing ARGs and MGEs compared to conventional composting. Mechanistically, this was likely driven by direct negative effects of the high temperature on the stability of ARGs and MGEs and direct or indirect negative effects on bacterial abundances and relative abundance of potential ARG-host bacteria. Our results also suggest that the relative importance of MGEs was more important in hyperthermophilic composting, while the role of the bacterial community composition was more important for conventional composting on ARG-removal. Hyperthermophilic composting thus represents a promising biotechnology for reducing the abundance of ARGs before solid waste land application.

632

633 **Supporting Information**

634 The temperature profile of two composting treatments; absolute abundances of ARG and MGE;
635 principal coordinate analysis; bacterial density and alpha diversity; taxonomic cladogram;
636 procrustes analysis; correlation between absolute ARG and MGE abundances; abundance of
637 potential ARG hosts; network analysis for patterns among ARGs and MGEs; redundancy analysis;

information of PCR primers; additional details on local similarity analysis.

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Notes

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Table 1 First-order kinetic model analysis showing the half-lives ($t_{1/2}$) and kinetic coefficients (k) for different ARGs and MGEs in hyperthermophilic (HT) and conventional (CT) composting.

Target ARG/MGE	HT			CT		
	$t_{1/2}$ (day)	k	R^2	$t_{1/2}$ (day)	k	R^2
<i>tetA</i>	2.43	0.33	0.84	/	/	/
<i>tetB</i>	1.20	0.70	0.89	2.98	0.39	0.57
<i>tetC</i>	1.27	0.55	0.99	1.58	0.52	0.99
<i>tetG</i>	1.59	0.49	0.88	2.85	0.54	0.93
<i>tetL</i>	0.098	2.58	0.50	0.12	10.95	0.69
<i>tetQ</i>	/	/	/	2.10	0.60	0.97
<i>tetO</i>	/	/	/	0.77	1.12	0.99
<i>tetX</i>	/	/	/	1.08	0.90	0.83
<i>sul1</i>	1.60	0.62	0.60	2.38	0.72	0.83
<i>sul2</i>	1.17	0.80	0.58	1.72	0.62	0.96
<i>sul3</i>	2.49	0.32	0.83	8.46	0.10	0.86
<i>strA</i>	1.64	0.53	0.73	2.46	0.49	0.94
<i>strB</i>	1.49	0.60	0.51	2.64	0.55	0.60
<i>aacA4</i>	1.35	0.54	0.96	3.80	0.41	0.88
<i>aadA</i>	1.60	0.61	0.67	2.59	0.62	0.91
<i>aadB</i>	2.15	0.47	0.70	1.90	0.58	0.91
<i>aadE</i>	1.18	0.62	0.98	3.15	0.43	0.97
<i>aphA1</i>	1.66	0.53	0.84	2.25	0.77	0.66
<i>ermB</i>	0.80	0.87	0.99	1.16	0.98	0.69
<i>ermT</i>	0.62	1.17	0.97	2.38	0.45	0.91
<i>ermX</i>	0.96	0.93	0.55	8.74	0.49	0.68
<i>mefA</i>	0.91	0.90	0.80	31.54	0.00	0.73
<i>ereA</i>	1.15	0.60	0.98	0.67	2.61	0.75

817	<i>intI1</i>	0.55	1.40	0.91	2.37	0.37	0.97
818	<i>Tn916</i>	1.01	0.76	0.89	1.70	0.48	0.99

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Note: For a better fitting model, first order kinetic mode ($t_{2/1}$) is based on data using absolute abundances of target genes from day 0 to 33.