Transcriptome Profiling of Cellular Response to Kanamycin B in *E.coli*

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Abstract

Background: Aminoglycosides are not only antibiotics but also have wider and diverse non-antibiotic cellular functions. No genome-wide study focusing on the changes of gene expression by aminoglycosides in *E.coli* has been reported. Here, we report transcriptome-profiling analysis of *E.coli* with or without Kanamycin B to elucidate the understanding of non-antibiotic cellular functions.

Results: The differentially expressed genes (DEGs) at two given concentrations of Kanamycin B were identified. The results indicated that Kanamycin B does not affect the expression of the majority of the genes. Functional classification of the DEGs revealed that they were mainly related to microbial metabolism including two-component systems, biofilm formation, oxidative phosphorylation and nitrogen metabolism in diverse environments.

Conclusions: Kanamycin B treatment causes diverse changes in the transcriptional profile of *E. coli* JM109, that are not directly associated with the antibiotic activity of Kanamycin B.

Background

Aminoglycosides are potent, broad-spectrum antibiotics that are usually known to inhibit translation by binding to the A site of the ribosome during the translocation step [1-3], they include: Kanamycin B, Gentamicin, Amikacin, Tobramycin, Neomycin, and Streptomycin. However, additional evidence is accumulating to suggest that the aminoglycosides can not only act as antibiotics that inhibit or stop bacterial growth but also have other wider and diverse cellular functions. The aminoglycosides are natural products synthesized through complex biosynthetic pathways [4], that can also act as modulators of bacterial growth [5, 6]. The aminoglycosides can bind to riboswitch RNA located at the 5' untranslated region of aminoglycoside acetyltransferase (AAC) or adenylyl transferase (AAD) to regulate translation of these resistance genes [7-11]. Various aminoglycoside binding sites have been identified in ribozymes [12], rRNA [1, 13], Rev responsive element [14] and HIV trans-activating region [15]. Aminoglycosides can enhance or inhibit known ribozyme activity [16-18]. Tobramycin is reported to induce the biofilm formation in bacteria [19]. Aminoglycosides are also involved in induction of the bacterial SOS response [20, 21]. Aminoglycoside binding RNA aptamers have been identified *in vitro* [22-25]. A Neomycin binding riboswitch selected *in vitro* was shown to regulate gene expression in *Saccharomyces cerevisiae* [26, 27]. Despite this collective evidence of the wide variety of non-antibiotic aminoglycoside functions and a report of Kanamycin B dependent changes in the transcriptional profile of *Mycobacterium tuberculosis* (*Mtb*) [28], no systematic study has focused on the genome-wide analysis how aminoglycosides alter gene expression in *E.coli*.

Here we have carried out transcriptome analysis by RNA-sequencing (RNA-seq) to investigate the genome-wide effect of Kanamycin B on *E.coli*. We identified the DEGs that are mainly related to microbial metabolism in diverse environments, two-component systems, nitrogen metabolism upon Kanamycin B

treatment. The results provide an insight into the wider non-antibiotic function of aminoglycosides and provide a foundation for a better understanding of aminoglycosides in clinical use.

Results

Overview of the RNA-seq data

To investigate the effect of Kanamycin B on *E. coli* JM109, we performed transcriptome-profiling analysis by RNA-seq in the absence or presence of Kanamycin B. Since Kanamycin B is a bactericidal antibiotic, initial cultures in the absence or presence of a broad range of Kanamycin B were cultivated to select concentrations that are neither lethal nor inhibit growth (Figure 1A). 0.5µM or 1µM of Kanamycin B showed minimal effects on cell growth, while 2µM of Kanamycin B clearly inhibited growth. As a result, 0.5µM and 1µM of Kanamycin B were chosen for transcriptome profiling analysis. Total RNA was extracted from cells at mid-log phase that had been treated with 0, 0.5µM and 1µM of Kanamycin B for RNA-seq analysis. The transcriptome-profiling data was uploaded to the National Center for Biotechnology Information Sequence Read Archive (accession number PRJNA756617). With three biological repeats of each sample, a total of 9 cDNA libraries were constructed containing 340.20 million raw reads; 339.64 million clean reads (accounting for 99.83% of raw reads) were recorded after removing adapter sequences and reads of low quality and those with more than 5% N bases. The average number of clean reads per sample was about 37.74 million and the clean Q20 (sequencing error rate < 1%) base rate was > 97.86% for each sample. Ultimately, 336.74 million high-quality reads (accounting for 99.14% of clean reads) were mapped to the *Escherichia coli* str. K-12 substr. MG1655 genome.

To investigate their reproducibility, the biological repeats were analysed by principal component analysis (PCA). The results revealed that PC1 and PC2 had values of 26.1% and 14.4%, respectively, and accounted for 40.5% of the principal components (Figure 1B). PCA showed consistency between the three replicates at each Kanamycin B concentration; samples from the biological repeat clustered together, reflecting minimal differences between them. PCA suggested that the three biological repeats in this study were reasonably reproducible (Figure 1B).

Gene expression profile in response to Kanamycin B treatment

The cellular response to Kanamycin B was revealed by the changes in the levels of gene expression. The differentially expressed genes (DEGs) in response to treatment by Kanamycin B were identified by selecting gene expression levels with |fold changes| ≥ 2 and significant differences in p-values of < 0.05 at 0.5 µM or 1 µM. Figure 2A shows a cluster analysis of the DEGs (for a full list of DEGs see additional file 1). There are two main clusters: one with genes that were induced and one with genes that were repressed in response to Kanamycin B treatment. We identified 83 or 136 induced DEGs and 112 or 155 repressed DEGs upon treatment with 0.5 µM or 1 µM of Kanamycin B respectively compared to no

treatment (Figure 2B). There are 65 upregulated and 58 downregulated DEGs in the 1 μ M / 0.5 μ M group (Figure 2B). Fewer numbers of DEGs were observed in the 1 μ M / 0.5 μ M group compared to the 0.5 μ M / 0 μ M and 1 μ M / 0 μ M groups suggesting that 0.5 μ M or 1 μ M Kanamycin B causes smaller cellular responses. We next compared the similarities and differences of the DEGs in response to 0.5 μ M or 1 μ M Kanamycin B treatment. The numbers of overlapping genes between the groups (0.5 μ M / 0 μ M, 1 μ M / 0 μ M and 1 μ M / 0.5 μ M) are shown in the Venn diagram for upregulated or downregulated DEGs (Figure 2C, 2D) and overlapping groups listed (additional file 2). There is considerable overlap between 0.5 μ M / 0 μ M and 1 μ M / 0 μ M DEG groups; 30 genes and 57 genes were co-induced or co-repress in 0.5 μ M and 1 μ M of Kanamycin B treatment. All of the DEGs represent 8.8% of the whole genome transcripts. In addition, the results show that the magnitude of the transcriptional responses in DEGs varied significantly from gene to gene. For example, some genes are upregulated over 10-fold upon Kanamycin B treatment while the expression of many other genes are induced only by about 2-fold (Table 1 and additional file 1).

Table 1

Functional classification of DEGs (1 μ M/0 μ M)

Gene Symbol	Fold change	Regulation	Location	Gene annotation
Microbial metabolism in diverse environments				
<i>nirD*</i>	219.45	up	cytosol	nitrite reductase subunit NirD
<i>nirB</i>	5.38	up	cytosol	nitrite reductase catalytic subunit NirB
<i>narI</i>	11.30	up	inner membrane	nitrate reductase A subunit γ
<i>narG</i>	9.58	up	inner membrane	nitrate reductase A subunit α
<i>narH</i>	7.48	up	inner membrane	nitrate reductase A subunit β
<i>ydhX</i>	3.61	up	periplasmic space	putative 4Fe-4S ferredoxin-like protein YdhX
<i>glcD</i>	3.09	up	cytosol	glycolate dehydrogenase, putative FAD-linked subunit
<i>glpE</i>	2.75	up	cytosol	thiosulfate sulfurtransferase GlpE
<i>yqeF</i>	2.39	up	cytosol	putative acyltransferase
<i>lysC</i>	2.14	up	cytosol	aspartate kinase III
<i>frdD</i>	-9.37	down	inner membrane	fumarate reductase membrane protein FrdD
<i>frdB</i>	-9.33	down	inner membrane, cytosol	fumarate reductase iron-sulfur protein
<i>frdA</i>	-6.35	down	inner membrane, cytosol	fumarate reductase flavoprotein subunit
<i>frdC</i>	-3.00	down	inner membrane	fumarate reductase membrane protein FrdC
<i>hyaB</i>	-4.88	down	periplasmic space, inner membrane	hydrogenase 1 large subunit
<i>hyaA</i>	-3.41	down	inner membrane	hydrogenase 1 small subunit
<i>adhE</i>	-2.86	down	cytosol	alcohol dehydrogenase/aldehyde-dehydrogenase
<i>adhP</i>	-2.67	down	cytosol	ethanol dehydrogenase / alcohol dehydrogenase
<i>gabT</i>	-3.19	down	cytosol	4-aminobutyrate aminotransferase GabT
<i>gabD</i>	-2.68	down	cytosol	succinate-semialdehyde dehydrogenase (NADP+) GabD
<i>gadA</i>	-2.43	down	cytosol	glutamate decarboxylase A
<i>gadB</i>	-2.10	down	cytosol, membrane	glutamate decarboxylase B

<i>hcaE</i>	-2.86	down	no annotation	putative 3-phenylpropionate/cinnamate dioxygenase subunit α
<i>paaK</i>	-2.73	down	cytosol	phenylacetate-CoA ligase
<i>yghX</i>	-2.57	down	no annotation	putative hydrolase fragment
<i>aldB</i>	-2.43	down	cytosol	aldehyde dehydrogenase B
<i>fbaB</i>	-2.19	down	cytosol	fructose-bisphosphate aldolase class I
<i>tktB</i>	-2.19	down	cytosol	transketolase 2
<i>allB</i>	-2.16	down	cytosol	allantoinase
Two-component system				
<i>fdnG</i>	19.98	up	periplasmic space	formate dehydrogenase N subunit α
<i>fdnH</i>	11.67	up	inner membrane	formate dehydrogenase N subunit β
<i>fdnI</i>	5.38	up	inner membrane	formate dehydrogenase N subunit γ
<i>narI</i>	11.30	up	inner membrane	nitrate reductase A subunit γ
<i>narG</i>	9.58	up	inner membrane	nitrate reductase A subunit α
<i>narH</i>	7.48	up	inner membrane	nitrate reductase A subunit β
<i>narJ</i>	6.84	up	cytosol	nitrate reductase 1 molybdenum cofactor assembly chaperone
<i>narX</i>	2.24	up	periplasmic space, inner membrane	sensory histidine kinase NarX
<i>rstB</i>	2.50	up	inner membrane	sensory histidine kinase RstB
<i>uhpB</i>	2.16	up	inner membrane	sensory histidine kinase UhpB
<i>baeS</i>	2.20	up	inner membrane	sensor histidine kinase BaeS
<i>rcaA</i>	3.35	up	cytosol	DNA-binding transcriptional activator RcsA
<i>ompF</i>	2.98	up	outer membrane	outer membrane porin F
<i>yqeF</i>	2.39	up	cytosol	putative acyltransferase
<i>uhpT</i>	2.19	up	inner membrane	hexose-6-phosphate:phosphate antiporter
<i>frdD</i>	-9.37	down	inner membrane	fumarate reductase membrane protein FrdD
<i>frdB</i>	-9.33	down	inner membrane, cytosol	fumarate reductase iron-sulfur protein
<i>frdA</i>	-6.35	down	inner membrane, cytosol	fumarate reductase flavoprotein subunit

<i>frdC</i>	-3.00	down	inner membrane	fumarate reductase membrane protein FrdC
<i>appB</i>	-6.19	down	inner membrane	cytochrome bd-II ubiquinol oxidase subunit II
<i>appC</i>	-4.33	down	inner membrane	cytochrome bd-II ubiquinol oxidase subunit I
<i>hyaC</i>	-7.15	down	inner membrane	hydrogenase 1 cytochrome subunit
<i>cusA</i>	-2.99	down	inner membrane	copper/silver export system RND permease
<i>mdtC</i>	-2.58	down	inner membrane	multidrug efflux pump RND permease subunit MdtC

Butanoate metabolism

<i>yqeF</i>	2.39	up	cytosol	putative acyltransferase
<i>frdD</i>	-9.37	down	inner membrane	fumarate reductase membrane protein FrdD
<i>frdB</i>	-9.33	down	inner membrane, cytosol	fumarate reductase iron-sulfur protein
<i>frdA</i>	-6.35	down	inner membrane, cytosol	fumarate reductase flavoprotein subunit
<i>frdC</i>	-3.00	down	inner membrane	fumarate reductase membrane protein FrdC
<i>gabT</i>	-3.19	down	cytosol	4-aminobutyrate aminotransferase GabT
<i>gabD</i>	-2.68	down	cytosol	succinate-semialdehyde dehydrogenase (NADP+) GabD
<i>gadA</i>	-2.43	down	cytosol	glutamate decarboxylase A
<i>gadB</i>	-2.10	down	cytosol, membrane	glutamate decarboxylase B
<i>adhE</i>	-2.86	down	cytosol	alcohol dehydrogenase/aldehyde-dehydrogenase
<i>dmlA</i>	-2.01	down	cytosol	D-malate/3-isopropylmalate dehydrogenase (decarboxylating)

Nitrogen metabolism

<i>nirD*</i>	219.45	up	cytosol	nitrite reductase subunit NirD
<i>nirB</i>	5.38	up	cytosol	nitrite reductase catalytic subunit NirB
<i>narK</i>	16.98	up	inner membrane	nitrate:nitrite antiporter NarK
<i>narI</i>	11.30	up	inner membrane	nitrate reductase A subunit γ
<i>narG</i>	9.58	up	inner membrane	nitrate reductase A subunit α
<i>narH</i>	7.48	up	inner membrane	nitrate reductase A subunit β
<i>hcp</i>	4.03	up	cytosol	protein S-nitrosylase

<i>can</i>	2.41	up	cytosol	carbonic anhydrase 2
Oxidative phosphorylation				
<i>cyoA</i>	2.48	up	inner membrane	cytochromebo3ubiquinol oxidase subunit 2
<i>ppa</i>	2.11	up	cytosol	inorganic pyrophosphatase
<i>frdD</i>	-9.37	down	inner membrane	fumarate reductase membrane protein FrdD
<i>frdB</i>	-9.33	down	inner membrane, cytosol	fumarate reductase iron-sulfur protein
<i>frdA</i>	-6.35	down	inner membrane, cytosol	fumarate reductase flavoprotein subunit
<i>frdC</i>	-3.00	down	inner membrane	fumarate reductase membrane protein FrdC
<i>appB</i>	-6.19	down	inner membrane	cytochrome bd-II ubiquinol oxidase subunit II
<i>appC</i>	-4.33	down	inner membrane	cytochrome bd-II ubiquinol oxidase subunit I
Alanine, aspartate and glutamate metabolism				
<i>gabT</i>	-3.19	down	cytosol	4-aminobutyrate aminotransferase GabT
<i>gabD</i>	-2.68	down	cytosol	succinate-semialdehyde dehydrogenase (NADP+) GabD
<i>gadA</i>	-2.43	down	cytosol	glutamate decarboxylase A
<i>gadB</i>	-2.10	down	cytosol, membrane	glutamate decarboxylase B
<i>aspA</i>	-2.49	down	cytosol	aspartate ammonia-lyase
<i>glsA</i>	-2.40	down	no annotation	glutaminase 1
Sulfur metabolism				
<i>ydhX</i>	3.61	up	periplasmic space	putative 4Fe-4S ferredoxin-like protein YdhX
<i>glpE</i>	2.75	up	cytosol	thiosulfate sulfurtransferase GlpE
<i>sbp</i>	2.05	up	periplasmic space	sulfate/thiosulfate ABC transporter periplasmic binding protein Sbp
<i>dmsB</i>	-4.23	down	inner membrane	dimethyl sulfoxide reductase subunit B
<i>dmsC</i>	-3.93	down	inner membrane	dimethyl sulfoxide reductase subunit C
<i>dmsA</i>	-2.49	down	inner membrane	dimethyl sulfoxide reductase subunit A
Arginine and proline metabolism				
<i>puuD</i>	3.81	up	cytosol	γ -glutamyl- γ -aminobutyrate hydrolase

<i>puuC</i>	3.22	up	cytosol	γ -glutamyl- γ -aminobutyraldehyde dehydrogenase
<i>puuA</i>	2.96	up	cytosol	glutamate-putrescine ligase
<i>speG</i>	2.31	up	cytosol	spermidineN-acetyltransferase
<i>patA</i>	-2.16	down	cytosol	putrescine aminotransferase
beta-Alanine metabolism				
<i>gabT</i>	-3.19	down	cytosol	4-aminobutyrate aminotransferase GabT
<i>gabD</i>	-2.68	down	cytosol	succinate-semialdehyde dehydrogenase (NADP+) GabD
<i>gadA</i>	-2.43	down	cytosol	glutamate decarboxylase A
<i>gadB</i>	-2.10	down	cytosol, membrane	glutamate decarboxylase B
Selenocompound metabolism				
<i>ynfE</i>	-9.15	down	periplasmic space, inner membrane	putative selenate reductase YnfE
<i>ynfF</i>	-5.26	down	periplasmic space, inner membrane	putative selenate reductase YnfF
<i>sufS</i>	-2.31	down	cytosol	L-cysteine desulfurase
<i>metE</i>	-2.15	down	cytosol	cobalamin-independent homocysteine transmethylase
Tyrosine metabolism				
<i>adhE</i>	-2.86	down	cytosol	alcohol dehydrogenase/aldehyde-dehydrogenase
<i>adhP</i>	-2.67	down	cytosol	ethanol dehydrogenase / alcohol dehydrogenase
<i>gabD</i>	-2.68	down	cytosol	succinate-semialdehyde dehydrogenase (NADP+) GabD
Chloroalkane and chloroalkene degradation				
<i>adhE</i>	-2.86	down	cytosol	alcohol dehydrogenase/aldehyde-dehydrogenase
<i>adhP</i>	-2.67	down	cytosol	ethanol dehydrogenase / alcohol dehydrogenase
Naphthalene degradation				
<i>adhE</i>	-2.86	down	cytosol	alcohol dehydrogenase/aldehyde-dehydrogenase

<i>adhP</i>	-2.67	down	cytosol	ethanol dehydrogenase / alcohol dehydrogenase
Nitrotoluene degradation				
<i>hyaB</i>	-4.88	down	periplasmic space, inner membrane	hydrogenase 1 large subunit
<i>hyaA</i>	-3.41	down	inner membrane	hydrogenase 1 small subunit
Polyketide sugar unit biosynthesis				
<i>rfbD</i>	2.97	up	cytosol	dTDP-4-dehydrorhamnose reductase
<i>rfbA</i>	2.48	up	cytosol	dTDP-glucose pyrophosphorylase
Taurine and hypotaurine metabolism				
<i>gadA</i>	-2.43	down	cytosol	glutamate decarboxylase A
Chlorocyclohexane and chlorobenzene degradation				
<i>yghX</i>	-2.57	down	no annotation	putative hydrolase fragment
<i>gadB</i>	-2.10	down	cytosol, membrane	glutamate decarboxylase B
Fluorobenzoate degradation				
<i>yghX</i>	-2.57	down	no annotation	putative hydrolase fragment
Inositol phosphate metabolism				
<i>appA</i>	-3.96	down	periplasmic space	periplasmic phosphoanhydride phosphatase/multiple inositol-polyphosphate phosphatase
Toluene degradation				
<i>yghX</i>	-2.57	down	no annotation	putative hydrolase fragment
Biosynthesis of ansamycins				
<i>tktB</i>	-2.19	down	cytosol	transketolase 2
Synthesis and degradation of ketone bodies				
<i>yqeF</i>	2.39	up	cytosol	putative acyltransferase

* The counts of the nirD gene is 0.

Functional classification of differentially expressed genes (DEGs)

To investigate the function of the DEGs, we carried out a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs. Table 1 (1 μM / 0 μM), additional file 3 (0.5 μM / 0 μM) and Figure 3 shows the DEGs classified into known or predicted functional groups. These genes encode proteins that are known or predicted to be involved in the following cellular functions: microbial metabolism in diverse environments; two-component systems, nitrogen metabolism, butanoate metabolism, Arginine and proline metabolism and Inositol phosphate metabolism. Genes that encode proteins that participate in cellular degradation, including chloroalkane and chloroalkene degradation, naphthalene degradation, chlorocyclohexane and chlorobenzene degradation, fluorobenzoate degradation, toluene degradation and degradation of aromatic compounds. Genes that encode proteins involved in oxidative phosphorylation, aminoacyl-tRNA biosynthesis or the TCA cycle. KEGG pathway analysis of DEGs showed that many genes are classified in more than one functional group (Table 1). Further analysis of the genes that belong to the five relatively large functional classes (two-component systems, oxidative phosphorylation, nitrogen metabolism, microbial metabolism in diverse environments and butanoate metabolism) revealed the linkage network of these DEGs (Figure 4, Table 1), suggesting an overlap between these main functional classes through these DEGs.

Series-cluster analysis and functional annotation of the clusters

In addition to the DEG analysis of the cellular response to Kanamycin B, we observed diverse and complex gene expression patterns over the Kanamycin B concentration courses. To dissect the gene expression patterns and further understand the gene functions, we performed a series-cluster analysis and functional annotation of the clusters. The series-cluster analysis can group genes that have similar expression patterns to form clusters of profiles and GO enrichment analysis further reveals functional annotations. A total of 4,534 genes were partitioned into 16 profiles of which 5 profiles were statistically significant ($p < 0.05$) (Figure 5, additional file 4). The Short Time-series Expression Miner (STEM) calculated the 5 profiles into 2 clusters. Each gene cluster exhibited a distinctive expression pattern. Cluster 1 includes profile 11 (115 genes), 12 (98 genes), 13 (107 genes) and 15 (116 genes). Genes in profile 11 show that expression levels increase rapidly after 0.5 μM Kanamycin B treatment and reach a plateau with 1 μM Kanamycin B, while the genes in profile 12, 13 and 15 present a gradual upregulation pattern. Cluster 2 consists of only profile 8 with 86 genes that exhibit similar expression from 0 to 0.5 μM Kanamycin B treatment and an increase in transcription with 1 μM Kanamycin B. STEM also supports GO enrichment analysis for each cluster and profile and includes functional classification of genes in the profiles in figure 5 (additional file 5). Profile 11 includes 115 genes of which 18 GO terms are associated with nitrogen compound metabolism and oxidation-reduction processes. The profile 12 cluster contains a number of membrane genes, and the bacterial outer membrane contributes to drug efflux resistance mechanisms. The profile 13 includes 107 genes enriched in 68 GO terms that are linked to DNA binding and the regulation of RNA biosynthesis. Profile 15 has 116 genes enriched in 10 GO Terms of which 8 are

related to biofilm formation and cell adhesion. Profile 8 in cluster 2 is associated with genes involved in RNA biosynthetic processes, regulation of primary metabolic process and cellular biosynthetic process.

Validation by real-time PCR (RT-PCR)

To verify the accuracy and reproducibility of the transcriptome results by an alternative method, we performed quantitation by RT-PCR. We selected 8 genes that were differentially expressed upon treatment with Kanamycin B. A comparative analysis of all the selected genes showed a similar expression pattern in the RT-PCR analysis as observed in RNAseq data (Figure 6). Thus, the RT-PCR experiments confirm the reliability of the transcriptome sequencing data.

Discussion

We set out to investigate the extra non ribosomal inhibitory cellular (i.e. the 'off target') function of Kanamycin B in *E. coli* through transcriptome data analysis (figure 1-4 and 6). DEGs and STEM analysis of transcriptome data revealed that Kanamycin B treatment effects the expression of genes that are involved in a variety of cellular functions including oxidative phosphorylation, nitrogen metabolism, microbial metabolism in diverse environments, biofilm formation and cell adhesion (table 1, figure 3-5).

It has been reported that the uptake of aminoglycosides into bacterial cells needs the proton motive force (PMF) that is produced by electron flow through the respiratory chain of oxidative phosphorylation. The PMF is mainly generated by the respiratory complex I that contains membrane proteins and/or Fe-S clusters and oxidoreductases [29]. The mechanism of aminoglycoside uptake and the bacterial cell response to the aminoglycosides is still unclear. Aminoglycoside binding riboswitches have been characterized [7] and a randomly selected Kanamycin B riboswitch has been reported [30]. The transcription levels of some genes associated with oxidative phosphorylation change upon treatment of Kanamycin B in this study. These observations together raise the possibility that Kanamycin B may regulate the expression of oxidative phosphorylation genes through riboregulatory interactions with the transcripts. Further studies will be required to examine this speculation.

In this study, treatment by Kanamycin B induces transcription of nitrate reductase (*narK*, *narG*, *narJ* and *narH*) and nitrite reductase (*nirD* and *nirB*) genes (Table 1), which are key genes that are involved in nitrogen metabolism. These reductases participate in the conversion of NO_3^- to NO_2^- to NO in cells and are associated with wider essential metabolic processes such as energy production, amino acid metabolism, biofilm formation, antibiotic resistance and bacterial pathogenesis. Nitrogen metabolism is also closely interlinked with biofilm formation that is also linked with the metabolism of glutamic acid, glutamine and arginine. It is widely recognized that NO plays an important role in modulating the architecture of biofilms [31, 32]. Other antibiotics have been reported to be able to induce biofilm formation [33]. Therefore, it is possible that Kanamycin B may induce the expression of genes associated with nitrogen metabolism through mimicking the effects of a natural ligand.

Genome-wide transcriptome profiling of *Mycobacterium tuberculosis* upon treatment of kanamycin has been reported [28]. Comparison with the functional classification and pathways of the DEGs identified in the *Mtb* study with those identified in this study indicated more differences than similarities between the two studies. This may be due to the following reasons: (1) *Mycobacterium tuberculosis* is a clinical strain that causes tuberculosis. The genotype of this strain may have evolved to escape the host immune system and the strain itself may have accumulated multiple mutations through the transmission of the tuberculosis. The genotype of the *Mtb* is fundamentally different from *E. coli* JM109 in our study. (2) The RNA-seq analysis with *Mtb* was treated with kanamycin, however the related aminoglycoside kanamycin B was used to treat the *E. coli* JM109 in this study. Although kanamycin B and kanamycin are analogue compounds, they also have a different resistance profile between the two organisms. The aminoglycoside riboswitch and many other regulatory RNAs can distinguish structural analogues through different binding affinities [7, 34, 35]. The RNA-seq is a sensitive technology that readily detects RNAs that respond to structural analogues. (3) The different growth culture conditions used in the *Mtb* study may also contribute to the different responses observed.

Conclusions

Transcriptome analysis of *E. coli* JM109 treated with kanamycin B uncovered gene expression networks that respond to kanamycin B. Kanamycin B treatment does not significantly effect the expression of the majority of genes. Functional classification of DEGs identified DEGs related to nitrogen metabolism, oxidative phosphorylation, amino acid metabolism and biofilm formation etc. The results provide insights into the cellular effects of Kanamycin B and are useful for establishing the non-antibiotic function of aminoglycosides Kanamycin B.

Materials And Methods

Bacterial strains and growth condition

The strain used in this study was *Escherichia coli* K-12 derived JM109 strain. JM109 was grown in rich medium LB at 0 μ M, 0.5 μ M, 1 μ M, 2 μ M Kanamycin B. OD600 was measured using a SpectraMax® M5.

RNA extraction and library preparation for transcriptome sequencing

E. coli JM09 was cultured with 0 μ M, 0.5 μ M, 1 μ M Kanamycin B to an OD600= 0.6. Total RNA of harvested cells was extracted using Trizol (Invitrogen, USA). The NEBNext Ultra II Directional RNA Library Prep Kit was used for the preparation of the library. The library preparations were performed following the manufacturer's protocol. Three biological replicates of each library were sequenced on an Illumina HiSeq X Ten (Illumina, San Diego, CA, USA) with 150-bp paired-ends by Genewiz (Suzhou, China). RNA-seq data

were uploaded to the Sequence Read Archive of the National Center for Biotechnology Information (accession number: PRJNA756617).

Sequence reads mapping and assembly

The raw reads of fastq format were initially filtered by removing reads containing adaptors, poly-N and low-quality reads. The high-quality clean reads were obtained (reads contain 20% base quality lower than Q20). Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. The analyses were performed by using high quality clean reads. The clean reads were subsequently mapped to the Escherichia coli str. K-12 substr. MG1655, genome assembly (NCBI: NC_000913.3) by using bowtie2 v2.2.6.

Principal Component Analysis (PCA) analysis

Principal Component Analysis (PCA) was performed to investigate the reproducibility of the biological repeats. DESeq2 in the deseq2_qc.r script was used for PCA calculation. The scores of the first and second principal components were plotted into two-dimensional space to represent the spatial relationships within the repeat samples for visualization [36].

Differential expression analysis (DEGs) and Functional annotations of DEGs

The expression levels of genes were counted by using HTseq in each sample [37]. The DEGs are filtered to meet the threshold level with a $|\text{fold change}| \geq 2$, and false discovery rate $p\text{-value} < 0.05$ in each pairwise comparison by using edgeR [38]. Functional annotations of DEGs are performed by R package, ClusterProfiler [39] with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Hierarchical clustering analysis

Hierarchical clustering analysis of differentially expressed genes was carried out by using the function heatmap.2 of gplots (version 3.0.1) in R version 3.3.1). The heatmaps are the outcome of this analysis to represent the reproducibility of the biological repeats [40].

Series-cluster analysis and functional annotation of the clusters

Short Time-series Expression Miner, version 1.3.13 (STEM) as a non-parametric clustering algorithm is designed to analyze short timeseries or concentration series expression data [41]. STEM is a new

clustering method that can identify real patterns from patterns. It clusters genes based on a series of pre-defined patterns (expression profiles). A profile is considered meaningful if the number of genes assigned to it surpasses the number of genes that are estimated to occur by chance. The functional annotations of the clusters were analyzed by Gene ontology (GO) that is imbedded in the STEM. GO enrichment analysis is considered significant if the p-value < 0.05.

Validation of DEGs by RT-PCR

The RNA samples for transcriptome sequencing were also used to perform real-time PCR to assess the reliability of the sequencing data. The RT-PCR reactions were carried out on a 7500 Real-Time PCR System (Applied Biosystems) using the SYBR® Premix Ex Taq™ II (Takara) according to the manufacturer's instructions. For absolute quantification, a gene-specific PCR amplicon of known concentration was prepared as a standard. The primers were obtained from Genewiz (Suzhou, China) (additional file 6).

Abbreviations

PCA: principal component analysis; DEGs: differentially expressed genes; KEGG: Kyoto Encyclopedia of Genes and Genomes; STEM: Short Time-series Expression Miner; RT-PCR: real-time PCR; PMF: proton motive force; GO: Gene ontology. *E. coli*: *Escherichia coli*; *Mtb*: *Mycobacterium tuberculosis*.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The RNA-Seq data supporting the results of this article have been uploaded to the Sequence Read Archive of NCBI (National Center for Biotechnology Information). It could be accessed via the NCBI SRA database with accession number PRJNA756617 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA756617/>).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Alastair Murchie and Dongrong Chen wrote the main manuscript text and Yaowen Chang and Xuhui Zhang prepared figure 1-6 and additional file 1-6. Yaowen Chang and Xuhui Zhang contributed equally. Alastair Murchie and Dongrong Chen are corresponding authors.

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Figures

Figure 1

Figure 1

E. coli growth curve and reproducibility of RNAseq data. a, Growth curve of JM109 treated with or without Kanamycin B. JM109 cells was grown in the presence of 0 μM, 0.5 μM, 1 μM, 2 μM Kanamycin B. Cell density was measured hourly. All data points were generated by three repeats. b, Principal component analysis of the RNA-Seq data. The small icon represents the original samples, colours are used to differentiate between treatments: red (0 μM), green (0.5 μM) and blue (1 μM).

Figure 2

Figure 2

Analysis of differentially expressed genes (DEGs) a, The hierarchical cluster analysis of 463 DEGs. Rows represent gene expression levels and columns represent concentration of Kanamycin b, treatment and repeats. The relative quantitative changes within the row are shown in colour: red suggests a relative higher expression whereas blue suggests a relative lower expression level. b, The number of DEGs in the comparison groups is presented in histogram. Upregulated DEGs (red), and downregulated DEGs (blue) were presented by histogram. c, The Venn diagram displays the number of the shared and unique genes of upregulated DEGs. d, The Venn diagram displays the number of the shared and unique genes of downregulated DEGs.

Figure 3

Figure 3

Functional classification of DEGs (0.5 μ M / 0 μ M and 1 μ M / 0 μ M) by KEGG enrichment analysis. The ordinate represents the name of the pathway; the diameter of the dots implies the gene ratio, ranging from 0.02 to 0.20 (smallest to biggest dot); the colour of the plot corresponds to P value.

Figure 4

Figure 4

Network representation of the five main enriched KEGG pathways and their associated DEGs in 2 comparisons (0.5 μM / 0 μM and 1 μM / 0 μM). Small circles represent genes involved whereas colors represent DEGs in different comparison. The large grey circles are the KEGG pathway, and the size of the circles reflect the p-value.

Figure 5

Figure 5

Cluster analysis of the gene expression patterns in the presence of 0 μ M, 0.5 μ M, 1 μ M of Kanamycin B. Based on the correlation coefficient the genes were assigned to the model profile that more closely matched its series. Five Profiles were significantly clustered ($P < 0.05$). The number of genes in each of the clusters and each profile is shown; grey line is the trend of individual gene expression; red line is the overall trend of gene expression.

Figure 6

Figure 6

RNA-seq data validation by RT-PCR. The blue histograms show the expression levels of 8 DEGs treated with 0.5 μM, 1 μM of Kanamycin B. The orange line indicates changes of gene expression levels upon treatment of different concentrations of Kanamycin B. Experiments were performed with three biological replicates. The right Y-axis indicates the fold change of RNA-seq data and the left Y-axis indicates the relative expression levels calculated by RT-PCR.

Supplementary Files

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