Proteomic analysis reveals that a global response is induced by subinhibitory concentrations of ampicillin

Lina Xiong, Dongjiang Liao, Xinpeng Lu, He Yan, Lei Shi, and Ziyao Mo

ABSTRACT
In this study, a recipient-donor co-culture system was used to research the effect of subinhibitory concentrations of antibiotics on horizontal transmission in bacteria and the influence of antibiotics on protein expression. We employed two-dimensional gel electrophoresis combined with mass spectrometry to compare the protein expression profiles in systems with or without 0.5 × the minimum inhibitory concentration of ampicillin. RT-PCR was used to assess the transcriptional levels of the differentially expressed genes. Fifty-seven different proteins were induced or suppressed. The upregulated proteins were involved in transcription and translation, cell wall synthesis, bacterial SOS response, and detoxifying functions, and the downregulated proteins were involved in metabolism. These results indicated that a global response was induced in the recipient-donor co-culture system by the subinhibitory concentration of ampicillin. Further analysis revealed that a global regulatory network based on key pathways was induced in the system in response to the antibiotic pressure. These findings provide a new, more comprehensive view for research on antibiotic-resistance mechanisms in recipient-donor co-culture.

KEYWORDS
ampicillin; antibiotic resistance; proteomics analysis; subinhibitory concentration

Introduction
The widespread use of antibiotics gives rise to the generation of antibiotic concentration gradients in the environment. Thus, bacteria are frequently exposed to subinhibitory concentrations of antibiotics, and recent evidence indicates that this is likely to have an important role in the dissemination and accumulation of resistance genes in bacteria.1-3 One of the main ways for the evolution of antibiotic resistance is the horizontal transfer of mobile genetic elements.4 Thus, researchers introduced a recipient-donor co-culture system (RDCS) to study the effect of subinhibitory concentrations of antibiotics on horizontal transfer among environmental microorganisms.5-7 In previous related studies, plating methods and molecular tests based on gene expression levels were generally used.5,7 However, it was difficult to obtain a complete picture of the effects of subinhibitory concentrations of antibiotics on the system and to understand the mechanisms underlying the observed effects.

Classical proteomic approaches provide valuable information about microorganisms. Proteomic methodologies, which can be used to analyze global changes in bacteria, have been used to study antimicrobial resistance mechanisms.8 It points out not only a few proteins that are directly related to an antibiotic resistance mechanism, but also a large number of differentially regulated proteins involved in various metabolic procedures, most of which are irrelevant to antibiotic used. Thus, it is difficult for researchers to find out the key proteins or mechanism. But then it contributes in the understanding of metabolic networks and their effect on antibiotic resistance. The changes in the protein expression patterns that occur in various microorganisms in response to β-lactam antibiotics have been studied.9,10 Nevertheless, to date, there are no published studies on the global effect of subinhibitory concentration of ampicillin (AMP) on a bacterial RDCS.

Here, we employed two-dimensional gel electrophoresis (2-DE) combined with MALDI TOF MS
spectrometry (MS) to compare the protein expression profiles in *Escherichia coli* RDCSs cultured with or without \(0.5 \times \) minimum inhibitory concentration (MIC) of AMP. The pathways involved were identified by bioinformatics. Finally, the transcriptional levels of the differentially expressed genes were validated by real-time reverse-transcription PCR (real-time RT-PCR). These findings provide a global view of a RDCS in the presence of subinhibitory concentrations of AMP from a proteomic perspective, and the discovery of relevant proteins may be helpful to develop new drugs which have synergistic effect on existing antibiotics and surmise the possible transmission mechanism of resistance gene.

**Results**

**MIC testing and measurement of growth rates**

The MIC of AMP was determined following the Clinical and Laboratory Standards Institute recommendations, and the MICs for the donor and recipient strains were both \(1 \mu g/mL\). To analyze the influence of subinhibitory concentrations of AMP on the RDCS, the system was treated with \(0.5 \times \) MIC AMP (0.5 \(\mu g/mL\)). Compared to the control, AMP had little effect on the bacterial growth rate (Fig. 1). Based on the growth profile, we identified the mid-logarithmic growth phase (at 6h, the donor/recipient ratios in RDCSs with and without AMP were both approximately 2.06) as the time point at which to harvest strains for protein extraction.

To gain insight into the physiological changes induced in the RDCS by a subinhibitory concentration of AMP, we compared the protein profiles in the RDCSs with and without AMP using 2-DE electrophoresis. The reproducibility of spots detected under the same experimental conditions was assessed by determining the number of spots and the level of overlap in replicate gels. After 6 h of culture with \(0.5 \times \) MIC of AMP, 59 protein spots were identified (Fig. 2).

**Protein identification**

The 59 spots were successfully identified as 57 different proteins by MS. To determine the functions of these proteins, we grouped them according to their functional categories. As shown in Table 1, in response to \(0.5 \times \) MIC of AMP, 33 proteins were upregulated, contained 5 proteins involved in transcription regulation, 7 in SOS repair, 3 in detoxifying functions, 3 translocators, 7 in metabolism regulation, 6 in peptidoglycan synthesis, and 2 hypothetical proteins of unknown function. However, one translocator, 22 proteins involved in metabolism regulation, and 1 in peptidoglycan synthesis were downregulated (Table 2).

**Transcriptional profiles of genes encoding differentially expressed proteins**

To validate the differential expressions of identified proteins, real-time RT-PCR analysis was performed. The total RNA from the untreated RDCS and the RDCS treated with \(0.5 \times \) MIC of AMP was extracted in parallel for more than three separate experiments. The mRNA levels of each gene in the control RDCS was arbitrarily set to one. The relative expression ratios of these genes between the AMP-treated RDCS and the control RDCS were shown in Supplementary file 1. These results confirmed that the changes of the gene transcriptions were in accord with those of the protein expressions.

**Protein-protein networks analyses**

In order to know the relationships among the proteins that were differentially expressed in the RDCS
in response to AMP, we analyzed the protein-protein interaction networks by using the STRING program (Version 10.0a.). As shown in Fig. 3a, the upregulated proteins from different pathways were linked to each other; however, the relationships among them were incompact. In contrast, most of the downregulated proteins were more closely related, creating an intricate web of associations.

Table 1. Identity of differentially expressed (upregulated) protein spots in the RDCS with 0.5 × MIC of AMP.

| Spot | Description | Protein | Accession number | MW* | pI | Score | Functional category |
|------|-------------|---------|------------------|------|----|-------|--------------------|
| 1    | Periplasmic binding protein/LacI transcriptional regulator | YcjW | gi|338768107 | 30935.4 | 6.85 | 191 | Transcription |
| 2    | D-ribose-binding periplasmic protein | RbsB | gi|3318867 | 28457.1 | 5.99 | 475 | Transcription |
| 3    | Ribosomal protein S2 | RpsB | gi|315616348 | 49725.3 | 9.57 | 458 | Transcription |
| 4    | 30S ribosomal protein S2 | RpsB | gi|26106512 | 23227.9 | 6.23 | 193 | SOS |
| 5    | Putative transcriptional regulator | GalS | gi|306815563 | 20357.1 | 7.85 | 107 | Transcription |
| 6    | Signal recognition particle | Ffh | gi|15988320 | 22110.6 | 5.68 | 481 | SOS |
| 7    | DNA protection during starvation protein | Dps | gi|331646031 | 23076.2 | 9.1 | 176 | SOS |
| 8    | Putative signal transduction protein | YjcC | gi|110643299 | 20535.7 | 7.85 | 107 | Transcription |
| 9    | Osmotically-inducible protein | OsmY | gi|331616006 | 19373.9 | 5.42 | 363 | SOS |
| 10   | Universal stress protein | UspD | gi|315616476 | 15939.3 | 6.03 | 173 | SOS |
| 11   | Universal stress protein | UspD | gi|15804512 | 16264.5 | 6.37 | 72 | SOS |
| 12   | FKB-type peptidyl-prolyl cis-trans isomerase | SylD | gi|15803860 | 34404.1 | 7.39 | 445 | SOS |
| 13   | Putative transcriptional regulator | GalS | gi|218701122 | 21864.9 | 5.84 | 363 | Detoxifying |
| 14   | DNA protection during starvation protein | Dps | gi|331646031 | 22110.6 | 5.68 | 481 | SOS |
| 15   | Putative signal transduction protein | YjcC | gi|110643299 | 20535.7 | 7.85 | 107 | Transcription |
| 16   | Osmotically-inducible protein | OsmY | gi|331616006 | 19373.9 | 5.42 | 363 | SOS |
| 17   | Universal stress protein | UspD | gi|315616476 | 15939.3 | 6.03 | 173 | SOS |
| 18   | Universal stress protein | UspD | gi|15804512 | 16264.5 | 6.37 | 72 | SOS |
| 19   | FKB-type peptidyl-prolyl cis-trans isomerase | SylD | gi|15803860 | 34404.1 | 7.39 | 445 | SOS |
| 20   | Modulator of drug activity | B | gi|74313566 | 21864.9 | 5.84 | 363 | Detoxifying |
| 21   | Glutathione S-transferase | GST | gi|15802049 | 22853.8 | 5.85 | 218 | Detoxifying |
| 22   | Chain A, Crystal Structure Of | The E. Coli Manganese Superoxide Dismutase Mutant Y174F | | | | | |
| 23   | PTS system, galactitol-specific II B component | GatB | gi|194436028 | 16995.9 | 5.3 | 225 | APH-sys |
| 24   | Sulfate ABC transporter, periplasmic sulfate-binding protein | CysP | gi|1991168981 | 37604.3 | 8.44 | 198 | ABC-trans |
| 25   | Glutamine transporter subunit | GlnH | gi|16128777 | 26714.1 | 6.08 | 166 | ABC-trans |
| 26   | Gluconate kinase | GmkA | gi|218998834 | 20787.6 | 5.97 | 352 | Metabolism |
| 27   | 3(‘2),5(‘3)-bisphosphate nucleotidase | CysQ | gi|188493062 | 27190.7 | 5.59 | 163 | Metabolism |
| 28   | Lipoamide synthase | LipA | gi|297521963 | 35261.9 | 7.79 | 77 | Metabolism |
| 29   | Nitrogenase family protein | MdA | gi|300947059 | 26351.7 | 6.45 | 313 | Metabolism |
| 30   | Quinone oxidoreductase, NADPH-dependent | YtfG | gi|26250832 | 35178.6 | 8.86 | 197 | Metabolism |
| 31   | ATP-dependent protease peptide subunit | HslV | gi|15804523 | 19081.1 | 5.96 | 139 | Metabolism |
| 32   | N-acetylmuramoylmeso-5-phosphate 2-epimerase | NanE | gi|157156014 | 24073.4 | 4.89 | 123 | PGN-syn |
| 33   | Aspartate carbamoyltransferase catalytic subunit | PyrB | gi|15804836 | 34457.7 | 6.12 | 105 | PGN-syn |
| 34   | Grotte phosphohistidine transferase | PyrE | gi|15804183 | 23522.2 | 5.33 | 110 | PGN-syn |
| 35   | Peptidoglycan-binding LysM | YgaI | gi|308089541 | 35717.7 | 9.42 | 90 | PGN-syn |
| 36   | Murine L,D-transportase | YnhG | gi|16129634 | 36059.9 | 9.42 | 372 | PGN-syn |
| 37   | Chain A, maltoporin maltose complex | LamB | gi|1941963 | 47355.2 | 4.72 | 633 | PGN-syn |
| 38   | Uropa1272 protein ycfP | YcfP | gi|222032861 | 21028.7 | 6.13 | 256 | Unknown |
| 39   | YbfU domain-containing protein | YbfU | gi|323973122 | 18964.2 | 6.06 | 493 | Unknown |

* Molecular weight; †, phosphotransferase system; ‡, ABC transport; §, peptidoglycan synthesis.
The comprehensive protein-protein network in the RDCS in response to AMP stress (Supplementary file 2) showed that the upregulated and downregulated proteins were likely coordinated and affected each other.

Table 2. Identity of differentially expressed protein spots (downregulated) in the RDCS with 0.5 × MIC of AMP.

| Spot | Description | Accession number | MW * | pl | Score | Functional category |
|------|-------------|------------------|------|----|-------|---------------------|
| 20   | Lysine-arginine-ornithine-binding periplasmic protein | ArgT gi | 26990.6 | 5.22 | 74 | ABC transporter |
| 21   | Class II aldolase, tagatose bisphosphate family | GatF gi | 32492.4 | 5.88 | 330 | Metabolism |
| 22   | 1-Phosphofructokinase | PfkB gi | 33752.3 | 5.37 | 265 | Metabolism |
| 23   | Glyceraldehyde-3-phosphate dehydrogenase | GapA gi | 21490.9 | 5.92 | 119 | Metabolism |
| 24   | Succinyl-CoA synthetase subunit alpha | SucD gi | 29758.5 | 6.32 | 694 | Metabolism |
| 25   | Succinate dehydrogenase, flavoprotein subunit | SdhA gi | 64397.1 | 5.85 | 668 | Metabolism |
| 26   | Fumarate hydratase (fumarase A), aerobic Class I | FumA gi | 60207.5 | 6.01 | 780 | Metabolism |
| 27   | Fructokinase | YnaA gi | 32484.2 | 4.98 | 510 | Metabolism |
| 28   | 2-Ketogluconate reductase | GhrB gi | 35302 | 5.5 | 101 | Metabolism |
| 31   | Transaldolase A | TalA gi | 35622.5 | 5.89 | 360 | Metabolism |
| 32   | DAHP+ synthase, tryptophan repressible | AroH gi | 38710.6 | 6.42 | 256 | Metabolism |
| 33   | L-Lactate dehydrogenase | LldD gi | 62727.7 | 5.12 | 144 | Metabolism |
| 35   | Alanine racemase | DaaX gi | 36021.3 | 6.8 | 137 | Metabolism |
| 36   | Bifunctional acetaldehyde-CoA/alcohol dehydrogenase | AdhE gi | 96096.7 | 6.32 | 146 | Metabolism |
| 37   | Cysteine synthase A | CysK gi | 34498.3 | 5.83 | 561 | Metabolism |
| 39   | Sulphite reductase (NADPH) hemoprotein, beta-component | CysJ gi | 63957.9 | 7.29 | 305 | Metabolism |
| 40   | Tryptophan synthase subunit A | TrpA gi | 24403.9 | 5.09 | 156 | Metabolism |
| 41   | Tryptophanase | TnaA gi | 52738.6 | 6.14 | 480 | Metabolism |
| 42   | Tryptophanase | TnaA gi | 52707.6 | 5.88 | 481 | Metabolism |
| 43   | D-Amino acid dehydrogenase, small subunit | DadA gi | 46966.5 | 6.03 | 144 | Metabolism |
| 44   | Acetylornithine deacylase | ArgE gi | 43730.1 | 5.54 | 551 | Metabolism |
| 45   | Ornithine carbamoyltransferase | Argl gi | 37240.7 | 5.45 | 96 | Metabolism |
| 47   | 3-Ketoacyl-CoA thiolase | FadA gi | 44512.2 | 9.11 | 161 | Metabolism |
| 51   | Chain A, E. coli glucosamine-6-P synthase in complex with glucose-6p and 3-oxo-L-norleucine | GlmS gi | 66721.6 | 5.56 | 107 | PGN-syn |

*, molecular weight; †, 3-Deoxy-D-arabino-heptulosonate-7-phosphate; ‡, peptidoglycan synthesis.

Discussion

AMP is a β-lactam antibiotic that can bind to transpeptidases and inhibit the biosynthesis of peptidoglycan in bacterial cell walls. To gain insight into the

(Fig. 3b). The comprehensive protein-protein network in the RDCS in response to AMP stress (Supplementary file 2) showed that the upregulated and downregulated proteins were likely coordinated and affected each other.
physiological changes in the RDCS exposed to subinhibitory concentration of AMP, we analyzed the expression of proteins of RDCS in the presence of 0.5 × MIC of AMP, and detected 57 proteins that were induced or suppressed. These proteins are involved in transcription and translation regulation, bacterial SOS response, detoxifying functions, substance transportation, metabolism, and peptidoglycan synthesis (Tables 1 and 2).

Five proteins, including YcjW, RbsB, RpsB, GalS, and Ffh, were identified as transcription and translation regulation proteins. Previous reports showed that upregulation of RbsB could promote the conjugation of plasmid in *E. coli* and RpsB was an important ribosomal protein for antibiotic resistance in gram-negative bacteria. In addition, our results also showed that two Lacl-type transcriptional regulators, YcjW and GalS, were involved in the response to AMP stress. Study has indicated that Lacl-type transcriptional regulator conserved among *Mycobacteria* and *Corynebacteria* that plays a role in the regulation of cell wall biogenesis. Considering the stabilizing mechanism of AMP, so we conjecture that upregulation of YcjW and GalS may be related with negative feedback. Thus, the results suggest that transcription and translation regulation are involved in the response to AMP stress, and the proteins involved may play a role in generation and spread of drug resistance.

Seven proteins related to the bacterial SOS response, LexA, Dps, YjcC, OsmY, UspG, UspD, and SlyD, were upregulated in the presence of AMP. Our finding of Dps as an upregulated protein is an only SOS protein consistent with an earlier report describing the genomic changes in wild type *E. coli* in response to 0.5 × MIC AMP. In this study, we used *E. coli* DH5α, a recA and gyrA deficient strain as a recipient. It may be the reason why unusually high number of SOS pathway genes being affected on ampicillin treatment despite that AMP does not cause direct DNA damage in *E. coli*.

Three proteins involved in oxidative stress response and detoxifying functions were upregulated, GST, MdaB, and SodA. It is generally known that oxidative stress induces most mutagenesis, and increased expression of Gst, MdaB, and SodA could protect *E. coli* from spontaneous mutagenesis.

Surprisingly, there were 30 differential proteins (50% of the total) that were involved in metabolic pathways, such as glycometabolism and amino acid metabolism. Among these proteins, 76.7% were decreased and 23.3% were increased in the presence of AMP. It is interesting to note that 92.3% of the proteins involved in the four most affected metabolic pathways (glycolysis, gluconeogenesis, the citrate cycle, and amino acid metabolism) were decreased; whereas only two proteins, GntK and GmhA, were increased (Fig. 4a). The upregulated GntK likely promoted the accumulation of sedoheptulose-7-phosphate, which is converted by GmhA to d-glycero-d-mannoheptose in the first step of lipopolysaccharide biosynthesis. In addition, many other metabolic pathways, such as lipoic acid, polypeptide, and protein metabolism, also appear to be involved in the response to AMP. Thus, in accordance with the results of Lin X *et al.*, who showed that downregulation of metabolic pathways is required for *E. coli* to respond to chlorotetacycline stress, proteins from most metabolic pathways were downregulated in the RDCS in the presence of subinhibitory concentrations of AMP. Based on the above findings, we conclude that fluctuations in metabolic pathways may be a tactic for antibiotic resistance. Thus, the relevant proteins could be used as drug targets for the development of synergistic antibiotics.

Additionally, four translocators were differential expressed; ArgT was downregulated, and the other three including GatB, CysP and GlnH were upregulated. GatB is one of the polypeptide chains of a galactitol-specific transporter that is involved in a signaling cascade that regulates transport, metabolism, and chemotaxis. CysP, GlnH, and ArgT are parts of three different ABC transporters involved in the uptake and metabolism of sulfate, glutamine, and lysine-arginine-ornithine, respectively. We speculate their expression fluctuations may be connected with fluctuations in metabolic pathways.

In another pathway, peptidoglycan synthesis, one protein was decreased and six were increased in the presence of AMP. Previous studies have indicated that YnhG and LamB are related with AMP resistance. Our study also showed that upregulation of NanE, PyrB, PyrE, YgaU and downregulation of GlmS probably facilitate peptidoglycan synthesis (Fig. 4b). Based on the antibacterial mechanism of AMP, fluctuations in this pathway indicate that negative feedback regulation plays an important role in the mechanism underlying defense against antibiotics.
The numerous changes in protein abundance observed in the RDCS in the presence of AMP suggested that various molecular changes in many metabolic pathways occur which allow the bacteria to adapt to adverse environmental conditions. In this study, 57 identifiable proteins were classified into six functional taxonomies. Among these functional taxonomies, the SOS response and detoxifying functions are known to be important for the response to adverse environmental conditions, and peptidoglycan synthesis may be associated with negative feedback regulation. The roles of the three other pathways are still unclear, and further experiments are needed to clarify their role in the response to AMP stress. However, it is noteworthy that metabolic products such as sedoheptulose-7-phosphate, fructosamine-6-phosphate, and ribose-5-phosphate are used as substrates in peptidoglycan biosynthesis. NADPH from the pentose pathway is required for reducing glutathione by GST to protect cells. Because of the ability of SodA and MdaB to reduce the bacterial mutation rate, their increased levels in AMP treatment RDCSs may be correlated with the increased abundance of Dps, which is classified as an SOS protein. In addition, decreased arginine synthesis (Fig. 4a) has been shown to slow cell growth and proliferation. As a result, the cell
growth rate falls below the maximal growth rate, which induces the expression of UspG and UspD. Based on the above findings, we deduce that the proteins from six functional taxonomies that are induced in the RDCS in response to AMP are not independent systems. Combined with our analysis of proteins network using STRING (Fig. 3 and Supplementary file 2), we conclude that there are multiple regulatory systems in the RDCS that functions cooperatively against antibiotics stress.

Since proteomic approaches provide information on protein species, but not the activity of these proteins, it is necessary to determine the roles of proteins in biological systems using relevant metabolomics and interaction studies.

**Conclusion**

In this study, analysis of the differential proteome showed that a global response is activated by *E. coli* in RDCSs in response to AMP, and this response includes upregulation of proteins involved in transcription and translation, SOS response, detoxifying functions, and cell wall synthesis and downregulation of proteins involved in metabolism. Our finding of these pathways associated with AMP resistance provides clear, accurate targets for new antibiotics. Moreover, our study also showed that a complex global regulation network based on several key pathways, including metabolic pathways, is involved in the response to stress in this RDCS. These findings provide a new, more comprehensive perspective for research on the mechanism of antibiotic resistance acquisition.

**Material and methods**

**Strains and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table 3. In the RDCS, *E. coli* BL21 carrying plasmid pR388 was used as the donor and *E. coli* DH5α carrying plasmid pACYC184-4 was used as the recipient.

Luria-Bertani (LB) broth (OXOID, UK) was used for bacterial growth and selective media included antibiotics as needed at the following concentrations: 50 mg/mL for chloramphenicol (CM), sulfamethoxazole (SMZ), and trimethoprim (TMP). Before coculture, the donor and recipient strains were cultured overnight at 37°C in Luria-Bertani (LB) broth supplemented with appropriate antibiotics, respectively. The bacterial cells were collected and washed three times in phosphate buffered saline for subsequent testing.

**Minimal inhibitory concentration (MIC) determination**

MICs were determined using the standard microdilution method recommended by the Clinical and Laboratory Standards Institute. Serial dilutions (216-0.125 μg/mL) of AMP were prepared in Müller-Hinton broth (Oxoid) in a volume of 50 μL per well in a 96-well plate, and then 50 μL of bacterial solution (1 × 10⁶ CFU/mL) was added. The plate was incubated for 18 h at 37°C without shaking. The lowest concentration of AMP without visible bacterial growth was defined as the MIC. MIC determinations were done in duplicate and as a minimum repeated thrice.

**Determination of the optimum duration of exposure to AMP**

The donor and the recipient strains were cultured overnight in LB broth supplemented with 50 μg/mL SMZ and TMP or 50 μg/mL CM, respectively. Collected cells from the overnight cultures were washed three times in phosphate buffered saline, and resuspended in LB broth (without antibiotics) and diluted to an OD₆₀₀ of ~0.1. Then mix up the donor suspension with the recipient suspension at the ratio of 2:1 quickly. Five milliliters of the mixture was transferred...
into two Erlenmeyer flasks containing 100 mL of LB broth (0.5 µg/mL of AMP were added into one of the flasks, and an equal volume of water was added to the other flask as a control), and the flask was incubated at 37°C with rotary shaking (200 rpm). At intervals of 2 h, the bacterial suspensions were serially diluted and plated in triplicate on LB-C, LB-ST, and LB agar plates supplemented with 50 µg/mL CM, SMZ, and TMP (LB-STC). Data represent the mean at least three biological replicates.

**Proteome analysis**

Cells were collected after 6 h of culture with either 0.5 µg/mL of AMP or water, and then washed with phosphate buffered saline three times. A portion of the collected cells was used for protein extraction, and the remaining cells were preserved at -80°C for subsequent total RNA purification. For the proteome analysis, 2-DE, gel image analysis, and MALDI TOF MS spectrometry were employed, as previously described. In brief, 2-DE was performed using pH 3-10 IPG gel strips (17 cm; Bio-Rad) and 12.5% SDS polyacrylamide gels in a Protean IEF Cell (Bio-Rad) with a Universal PowerPac (Bio-Rad). The gels were stained with silver and analyzed using ImageMaster 2D Elite 5.0 software (GE Healthcare). This program grouped the gel images into two classes containing biological triplicates of each condition: EC-C (RDCS without AMP) and EC-A (RDCS with 0.5 µg/mL of AMP).

MALDI TOF MS spectrometry analysis was conducted using a 4800 plus MALDI TOF/TOF Analyzer (Applied Biosystems) according to the method of Rao et al. The search parameters were as follows: taxonomy restrictions to Bacteria and trypsin digestion with one missed cleavage allowed. Proteins with a score above the threshold (p < 0.05) were considered positive. Proteomics experiments were repeated six times.

**RT-PCR**

Total RNA extraction and reverse transcription were performed using the RNAiso Plus Kit and PrimeScript<sup>TM</sup> RT reagent Kit, respectively, according to the manufacturer’s (Takara) instructions. The primers used are shown in Supplementary file 3. The E. coli 16S rRNA gene was amplified as the internal control. The RT-PCR protocol and reaction mixture, which included SYBR® Premix Ex Taq™ II (Takara), were according to the manufacturer’s instructions. The quantitative PCR data were analyzed using the relative expression software tool REST2009 v2.0.13.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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