Homeostasis of Glutathione Is Associated with Polyamine-Mediated β-Lactam Susceptibility in Acinetobacter baumannii ATCC 19606

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Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl–glycine) thiol compound existing in many bacteria and maintains a proper cellular redox state, thus protecting cells against toxic substances such as reactive oxygen species. Polyamines (spermine and spermidine) are low-molecular-weight aliphatic polycations ubiquitously presenting in all living cells and modulate many cellular functions. We previously reported that exogenous polyamines significantly enhanced β-lactam susceptibility of β-lactam-associated multidrug-resistant Acinetobacter baumannii. In this study, three genes differentially associated with the polyamine effects on β-lactam susceptibility were identified by transposon mutagenesis of A. baumannii ATCC 19606. All three genes encoded components of membrane transport systems. Inactivation of one of the genes encoding a putative glutathione transport ATP-binding protein increased the accumulation of intracellular glutathione (∼150 to ∼200%) and significantly decreased the polyamine effects on β-lactam susceptibility in A. baumannii ATCC 19606. When the cells were grown with polyamines, the levels of intracellular glutathione in A. baumannii ATCC 19606 significantly decreased from ∼0.5 to ∼0.2 nmol, while the levels of extracellular glutathione were correspondingly increased. However, the levels of total glutathione (intra- plus extracellular) were unchanged when the cells were grown with or without polyamines. Overall, these results suggest that exogenous polyamines induce glutathione export, resulting in decreased levels of intracellular glutathione, which may produce an improper cellular redox state that is associated with the polyamine-mediated β-lactam susceptibility of A. baumannii. This finding may provide a clue for development of new antimicrobial agents and/or novel strategies to treat multidrug-resistant A. baumannii.

Acinetobacter baumannii is a major hospital-acquired human pathogen causing a variety of diseases, including ventilator-associated pneumonia (1). Treatment of these pathogens involves the use of one or more antibiotics, typically β-lactams, aminoglycosides, or quinolones. Combination therapy is recommended to reduce the risk of antibiotic resistance and enhance the eradication rate (1, 2). Despite the use of combination therapy, treatment is very difficult due to the organism’s innate resistance and enormous capability to acquire resistance to commercially available antibiotics, which is reflected in numerous reports of multidrug-resistant clinical isolates of A. baumannii (1–5).

Glutathione is a tripeptide (L-γ-glutamyl–L-cysteinyl–glycine) thiol compound and accounts for >90% of the total nonprotein sulfur in bacteria. Some prokaryotic cells produce other low-molecular-weight thiols, which appear to function similarly to glutathione (6, 7). Glutathione is synthesized by two sequential ATP-dependent reactions catalyzed by γ-glutamyl-cysteine synthetase, encoded by gshA, and glutathione synthetase, encoded by gshB. γ-Glutamyl-cysteine synthetase catalyzes the formation of a peptide bond between the γ-carboxyl group of glutamate and the α-amino group of cysteine. Glutathione synthetase forms a peptide bond between the α-carboxyl of cysteine in γ-glutamyl-cysteine and the α-amino group of glycine. Glutathione synthesis is increased during early stationary growth and tightly regulated by feedback inhibition of γ-glutamyl-cysteine synthetase (8, 9). The thiol (sulphydryl) group of glutathione is responsible for its biological activity and is involved in a variety of functions, such as maintenance of the proper redox state in the cytoplasm, regulation of cellular function by glutathionylation, regulation of intracellular potassium levels, deactivation of toxic substances, and adaptation to stresses such as oxidative stress, temperature stress, and osmotic stress in bacteria (10, 11).

Glutathione was also observed in bacterial culture media and used for amino acid sources (8, 12). The γ-linkage between the glutamate and cysteine of glutathione protects the tripeptide from degradation by intracellular peptidases. However, the glutathione can be cleaved into a γ-glutamyl residue and L-cysteinyl-glycine by the γ-glutamyl transpeptidase localized outside the plasma membrane. Subsequently, the L-cysteinyl-glycine is cleaved into cysteine and glycine by dipeptidase. The three amino acids, and particularly cysteine, can be transported into the cell (10, 11, 13, 14). Cysteine is very unstable and rapidly auto-oxidizes to cystine. If intracellular concentrations of cysteine increase, the amino acid can be involved in the reduction of trivalent iron due to catalysis by the Fenton reaction in the presence of H₂O₂ to form the toxic hydroxyl radical (15, 16). Therefore, intracellular cysteine must be kept a very low levels (<200 μM) under normal growth condition (17); glutathione is one of such nontoxic reserve forms. Overall, these pathways suggest that certain levels of intracellular and extracellular glutathione are important in bacteria. A glutathione importer system has been reported for Escherichia coli (18), but little is known about glutathione exporter systems in bacteria.

Polyamines (spermine and spermidine) are essential intracellular cationic compounds found in all living organisms (19, 20). Since polyamines are cationic compounds, they interact with neg-
atively charged macromolecules (e.g., nucleic acids, proteins, and phospholipids) and are involved in numerous cellular functions, such as virulence (21), acid resistance (22, 23), biofilm formation (24), free radical scavenging (25), and iron scavenging (26), and as signal molecules (27). We previously reported that exogenous polyamines enhanced susceptibility to some β-lactam antibiotics in A.baumannii and Pseudomonas aeruginosa (28–30). However, the molecular details of this polyamine effect on β-lactam susceptibility remain unclear. In this study, three genes associated with the polyamine effect on β-lactam susceptibility were identified by transposon mutagenesis of A. baumannii ATCC 19606. One of the genes encoded a putative glutathione transport ATP-binding protein; this finding was used to confirm its involvement in polyamine-mediated β-lactam susceptibility in A. baumannii ATCC 19606.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemicals. A. baumannii ATCC 19606 (American Type Culture Collection, Manassas, VA) was used for transposon mutagenesis and mutant library construction. E. coli DH5α was used as a host strain for general cloning purposes. Bacterial strains were routinely grown on Luria-Bertani (LB) agar plates or broth at 37°C. All antibiotics, polyamines (spermine tetrahydrochloride and spermidine trihydrochloride), reduced glutathione, and other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO), and appropriate concentrations of them were supplemented in the culture media when needed.

Transposon mutagenesis and library construction. Transposon mutagenesis of A. baumannii was performed as described previously (31). Briefly, a culture of A. baumannii grown overnight was diluted 1/100 in LB broth and grown to a cell density of about 2.0 × 10^9 cells/ml (late exponential phase). The cells were collected by centrifugation (5,000 rpm) and washed once in ice-cold double-distilled water (ddH2O). The cells were washed three times in ice-cold 10% glycerol and resuspended in 10% glycerol to a final concentration of about 2.0 × 10^10 cells/ml. These electrocompetent cells were electroporated by the use of the EZ-Tn5 (R6K<sub>ori</sub>-KAN-2) Tnp transposome (Epicentre Biotechnologies, Madison, WI). Electroporation was performed by using a Micro Pulser (Bio-Rad, Hercules, CA) at preprogrammed settings of 2.5 kV (0.2-cm cuvette) with one pulse. Transformed colonies were selected on agar plates containing kanamycin (50 μg/ml). Individual transformed colonies were stored in a freezer (−80°C) for further analysis.

Rescue cloning and DNA sequencing. Genomic DNA extracted from the transformed colonies was digested by EcoRI and self-ligated. The ligation mixture was used to transform electrocompetent pir-116 E. coli cells as suggested by Epicentre Biotechnologies. Rescued plasmids were selected on agar plates containing kanamycin (50 μg/ml) and purified to determine flanking DNA sequences of the insertion cassette.

Antibiotic susceptibility testing. Antibiotic susceptibility was determined by Micro, guided by Clinical and Laboratory Standards Institute (CLSI) guidelines (28). Briefly, serial 2-fold dilutions of antibiotics were performed by using Mueller-Hinton (MH) broth (pH 7.0) supplemented with spermine (1 mM) or spermidine (10 mM) or without any extra compound. Fresh cultures of each mutant or parental strain grown overnight were diluted in saline to an optical density at 600 nm (OD<sub>600</sub>) of 0.1 to 0.12 (approximately 10<sup>6</sup> CFU/ml), and a portion of the adjusted cell suspension (5 μl for ~10<sup>7</sup> CFU) was inoculated. The cell cultures were incubated overnight (18 to 20 h) at 37°C. The MIC was defined as the lowest concentration of each antibiotic that completely inhibited the growth of the inoculum. MIC measurements were repeated and confirmed in three independent experiments.

Genetic complementation. A full-length gene encoding a putative glutathione transport ATP-binding protein (homolog of AB57_1837 [2,100 bp] of A. baumannii AB0057) from A. baumannii ATCC 19606 was amplified by PCR methods (32) and inserted into the TOPO TA cloning vector (Invitrogen, Grand Island, NY). For genetic complementation, a full-length gene from the TA cloning vector was inserted into a modified shuttle vector for A. baumannii (pWH1266-blaC:Tc). The modified shuttle vectors carrying the full-length gene were used to transform A. baumannii ATCC 19606 harboring the knocked-out gene.

Glutathione assay. Intracellular and extracellular levels of total glutathione (reduced and oxidized) were measured as described previously by Akerboom and Sies (33), using commercial glutathione assay reagents (Sigma-Aldrich, St. Louis, MO). Briefly, cells cultured overnight were diluted (1/100) into fresh LB broth (50 ml), and the diluted cells were grown at 37°C with shaking (250 rpm). An aliquot of cells (2 ml) was collected when the OD<sub>600</sub> reached ~0.5 (for exponential growth), ~1.0 (for late exponential growth), ~1.5 (for early stationary growth), and ~1.75 (for stationary growth). Half of the aliquot (1 ml) was filtered through a membrane (pore size, 0.2 μm; EMD Millipore, Billerica, MA), and the other half of cells was used to measure intracellular and extracellular levels of glutathione after breaking the cells by sonicication (Misonix Sonicators, Newtown, CT) (37% amplitude [15 W] for 10 s 10 times, with 30 s of cooling between bursts, until the cell extract was clear). The crude extract of cells and supernatant was spun down at 15,000 rpm for 5 min, and the supernatant was used to measure levels of glutathione. Glutathione levels in the samples were measured by using a kinetic assay in which catalytic amounts of glutathione cause a continuous reduction of 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) to 5-thio-2-nitrobenzoic acid (TNB), and the product (TNB) was assayed calorimetrically at 412 nm by using a DU 730 UV-visible spectrophotometer (Beckman Coulter, Inc., Indianapolis, IN). Glutathione standard solutions were used to calculate amount of glutathione, and the calculation (nmol glutathione per ml of sample) was performed according to the following equation: ([ΔA<sub>413</sub>/min [sample] × dilution factor of original sample]/[ΔA<sub>413</sub>/min [1 nmol] × volume of sample in the reaction mixture in ml]).

RESULTS AND DISCUSSION

Isolation and identification of mutant strains lacking polyamine-mediated β-lactam susceptibility. The MIC of carbenicillin (64 μg/ml) was decreased up to 8- or 16-fold (MICs of 8 or 4 μg/ml in A. baumannii ATCC 19606 when carbenicillin was combined with polyamines (spermine or spermidine) for MIC measurements (30). To identify the genetic determinants associated with the polyamine effect on carbenicillin susceptibility, a mutant library of A. baumannii ATCC 19606 was generated by the EZ-Tn5 (R6K<sub>ori</sub>-KAN-2) Tnp transposome (Epicentre Biotechnologies, Madison, WI), as described in Materials and Methods. Five hundred colonies from the mutant library were used to identify mutant strains lacking the polyamine effect. Three mutant strains (M1, M2, and M3) of A. baumannii showed decreased MICs of carbenicillin, from 256 μg/ml to 128 or 32 μg/ml, when combined with the polyamines for MIC measurements. One of the mutant strains (M3) was consistently less susceptible to aztreonam and carbenicillin when combined with the polyamines for MIC measurements (Table 1).

Genomic DNA was extracted from the three mutant strains of A. baumannii and used to isolate clones carrying the transposon-integrated DNA sequences by a rescue cloning method, as described in Materials and Methods. Three rescue clones, pBA60 for M1, pBA70 for M2, and pBA71 for M3, were isolated, and flanking nucleotide sequences of the transposon were compared to the entire genome sequence of A. baumannii AB0057 (GenBank accession number CP001182). Results revealed that the transposon was integrated at nucleotides 2751190 in mutant strain M1, 3400120 in mutant strain M2, and 1925894 in mutant strain M3. Inactivated genes were AB57_2663, encoding an RND family efflux.
transporter for mutant strain M1; AB57_3299, encoding conserved hypothetical transporter protein for mutant strain M2; and AB57_1837, encoding a putative glutathione transporter ATP-binding protein for mutant strain M3. An intact AB57_1837 gene fully restored the polyamine effects in the mutant strains of A. baumannii (Table 1). These results indicate that the AB57_1837 gene is associated with polyamine-mediated β-lactam susceptibility in A. baumannii.

We previously reported that exogenous polyanilines enhanced the susceptibility to β-lactam antibiotics of several bacteria, including A. baumannii (28, 30). We also excluded an involvement of outer membrane permeability or rupture, β-lactamase activity or ampC, efflux pump (AcrAB), porin protein (OprD), and lipopolysaccharide (LPS) in the polyamine effects on β-lactam antibiotics (28–30). In this report, we successfully isolated three mutant strains of A. baumannii lacking the polyamine effect by using a transposon-integrated mutant library. One of the mutated genes, encoding a putative glutathione transporter ATP-binding protein, was confirmed to be involved in the polyamine effect in A. baumannii ATCC 19606. Interestingly, all mutated genes encoded components of membrane transport systems, and the polyamine effects were differentially abolished in the mutant strains. These results suggest that the polyamine effects may be associated with unbalanced intracellular (or extracellular) solutes employed by the membrane transport systems.

Glutathione production and transport. Glutathione is synthesized in the cytoplasm, and significant amounts are exported into the culture medium (8, 12). The AB57_1837 gene encodes a putative glutathione transporter ATP-binding protein in A. baumannii, suggesting that the mutant strain for the gene may have alterations in glutathione transport. To examine whether glutathione is synthesized in A. baumannii and whether the gene is involved in glutathione transport, intracellular and extracellular levels of glutathione were measured as described in Materials and Methods. In A. baumannii ATCC 19606, intracellular levels of glutathione were about 0.2 nmol during exponential growth (OD600 of ~0.5) and increased up to about 0.8 nmol during early stationary growth (OD600 of ~1.5), but glutathione was not always detectable during stationary growth (OD600 of ~1.75). Extracellular glutathione was always detected, and the levels were increased up to about 1 nmol during early stationary growth and then decreased (Fig. 1). In the mutant strain of A. baumannii ATCC 19606 (a homologue of AB57_1837::Tn5-Km), intracellular levels of glutathione at exponential, late exponential (OD600 ~1.0), and early stationary growth phases were higher (~150 to ~200%) than those in the parental strain (Fig. 2), but extracellular levels of glutathione were slightly lower than those in the parental strain (data not shown).

The results revealed that A. baumannii synthesized substantial levels of glutathione and that significant amounts of glutathione were exported into the culture medium, similar to the patterns reported previously (8, 12). In bacteria, the levels of glutathione synthesis are highly diverse and range from no production to about 8 μmol in bacteria (7). The mutant strain accumulated significant amounts of intracellular glutathione, with decreased levels of extracellular glutathione. These results suggest that the mutated genes are associated with alterations of glutathione transport. The AB57_1837 gene is likely cotranscribed with the genes encoding permease and a probable solute-binding protein com-

**TABLE 1 Polyamine-mediated β-lactam susceptibility in A. baumannii**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)*</th>
<th>Aztreonam with:</th>
<th>Carbenicillin with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>Spn Spd</td>
</tr>
<tr>
<td>A. baumannii ATCC 19606</td>
<td>64</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>A. baumannii ATCC 19606 M3 (AB57_1837::Tn-Km)*</td>
<td>128</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>A. baumannii ATCC 19606 M3 (AB57_1837::Tn-Km)/pBA83*</td>
<td>64</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>A. baumannii ATCC 19606/pWH1266-bla::Tc (cloning vector)</td>
<td>64</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

* MIC measurements were repeated three times, with identical results. Spn, spermine (1 mM); Spd, spermidine (10 mM).

* The AB57_1837 gene is from A. baumannii AB0057.

* pBA83 carries an intact gene, a homolog of AB57_1837 of A. baumannii AB0057, from A. baumannii ATCC 19606.
ponent of the ABC transporter in A. baumannii (http://www.ncbi.nlm.nih.gov/). The glutathione export system is currently uncharacterized in bacteria, and the gene may be a component of the glutathione export system in A. baumannii.

Polyamine effects on glutathione transport. Intracellular and extracellular levels of glutathione were measured in A. baumannii ATCC 19606 cells grown with or without polyamines to investigate the effects of polyamines on glutathione transport. As shown in Fig. 3, extracellular (supernatant) levels of glutathione were significantly increased (from about −0.8 to −1.3 nmol) in the cells grown with polyamines compared to those in the same cells grown without polyamines. In contrast, intracellular (cell pellet) levels of glutathione were correspondingly decreased (from about −0.5 to −0.2 nmol) in the cells grown with polyamines compared to the same cells grown without polyamines. However, total glutathione (intra- plus extracellular) levels were similar in cells grown with and without polyamines.

The results demonstrate that exogenous polyamines enhance glutathione export into the culture medium without increasing (or decreasing) glutathione synthesis and result in decreased levels of intracellular glutathione with correspondingly increased levels of extracellular glutathione. Polyamines are cationic compounds associated with a variety of cellular functions, including gene expression. For example, polyamines induce phoPQ and subsequently an operon of LPS modification, resulting in resistance to cationic antibiotics in P. aeruginosa (34). Polyamines are also involved in gene expression at the level of translation (35, 36). This observation suggests that polyamines may cause upregulation of the glutathione export system(s) at the transcription and/or translation level, resulting in decreased levels of intracellular glutathione. Prevention of glutathione export abolished the polyamine effect on β-lactam antibiotics in A. baumannii. These results indicate that lower levels of intracellular glutathione may be required for polyamine-mediated β-lactam susceptibility. Glutathione is a multifunctional thiol compound that plays a critical role in maintaining a proper cellular redox state such that protein thiols with a proper redox state can protect cells against reactive oxygen species, reactive nitrogen species, and reactive electrophilic species (37). It was also reported that some antibiotics, including β-lactams, induce highly deleterious hydroxyl radicals in bacteria (38). Therefore, increased levels of hydroxyl radicals induced by β-lactam antibiotics may enhance β-lactam susceptibility at low levels of intracellular glutathione caused by the polyamines. Overall, the results lead to the conclusion that exogenous polyamines induce glutathione export by producing an improper cellular redox state, which may be associated with polyamine-mediated β-lactam susceptibility.

ACKNOWLEDGMENT

This work was supported by a grant from the National Institutes of Health (S5C3 GM094053).

REFERENCES


