Lysozyme and Penicillin Inhibit the Growth of Anaerobic Ammonium-Oxidizing Planctomycetes

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Anaerobic ammonium-oxidizing (anammox) planctomycetes oxidize ammonium in the absence of molecular oxygen with nitrite as the electron acceptor. Although planctomycetes are generally assumed to lack peptidoglycan in their cell walls, recent genome data imply that the anammox bacteria have the genes necessary to synthesize peptidoglycan-like cell wall structures. In this study, we investigated the effects of two antibacterial agents that target the integrity and synthesis of peptidoglycan (lysozyme and penicillin G) on the anammox bacterium Kuenenia stuttgartiensis. The effects of these compounds were determined in both short-term batch incubations and long-term (continuous-cultivation) growth experiments in membrane bioreactors. Lysozyme at 1 g/liter (20 mM EDTA) lysed anammox cells in less than 60 min, whereas penicillin G did not have any observable short-term effects on anammox activity. Penicillin G (0.5, 1, and 5 g/liter) reversibly inhibited the growth of anammox bacteria in continuous-culture experiments. Furthermore, transcriptome analyses of the penicillin G-treated reactor and the control reactor revealed that penicillin G treatment resulted in a 10-fold decrease in the ribosome levels of the cells. One of the cell division proteins (Kustd1438) was downregulated 25-fold. Our results suggested that anammox bacteria contain peptidoglycan-like components in their cell wall that can be targeted by lysozyme and penicillin G-sensitive proteins were involved in their synthesis. Finally, we showed that a continuous membrane reactor system with free-living planktonic cells was a very powerful tool to study the physiology of slow-growing microorganisms under physiological conditions.

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activity in an anammox culture and reported that the compound had no effect on anammox bacteria (26). Nevertheless, both of these studies were performed on enrichment cultures with less than 80% anammox bacteria growing as biofilm aggregates, which could have contained penicillin-degrading microorganisms or provided protection against antibiotics as a physical barrier. Further, they were conducted either as batch experiments (25) or by adding penicillin with long intervals without considering the fate of the added penicillin (26). The long-term effect of lysozyme on anammox bacteria has not been tested yet.

Surprisingly, a recent metagenomic analysis indicated that the anammox organism *Kuenenia stuttgartiensis* encodes 19 out of 21 genes that are necessary for peptidoglycan biosynthesis. Two of these (*kustal1895* and *kust3276*) encode proteins that are homologous to enzymes that are suggested to be monofunctional (transpeptidase) PBP s (27). The absent two genes are homologous to PBP 1a and PBP 1b in *Escherichia coli*, which have both transglycosylase and transpeptidase activities and which have been suggested to be essential for cross-linking of sugar monomers (16). It should be noted that the reports on the function of both classes of proteins are derived from studies with laboratory strains that are not genetically related to anammox bacteria (e.g., *E. coli*), and the function of proteins cannot be inferred directly through sequence comparison.

Evidence from comparative genomic analysis of *K. stuttgartiensis* and other planctomycetes as well as Gram-negative bacteria also suggested that anammox bacteria could be genetically able to possess a Gram-negative bacterium-like cell wall structure (28). Nevertheless, this hypothesis cannot be supported by ultrastructural studies since the peptidoglycan layer, which was usually clearly visible in thin sections of Gram-negative bacteria, could not be observed in thin sections of anammox bacteria (29). Consequently, whether anammox bacteria have a cell wall containing a peptidoglycan-like component or not is still unknown.

In the present work, we studied the effects of penicillin G and lysozyme on *K. stuttgartiensis*. To this end, we used a highly enriched free-living planktonic cell culture (>95% enriched) in batch tests and continuous membrane reactors that recently became available (1, 30). Streptomycin, a type of aminoglycoside antibiotic (protein synthesis inhibitor) targeting the 30S subunit of the bacterial ribosome (31), was also used as a positive control.

**MATERIALS AND METHODS**

**Genome analyses.** All translated gene sequences of anammox species *K. stuttgartiensis* (27), *Brocadia fulgida* (32), and *Scalindua profunda* (33) were directly downloaded from the genome database at NCBI, JGI, or IMG/ML. Downloaded sequences were submitted to the KEGG Automatic Annotation Server (KAAS) (34) for pathway mapping. Proteins of *K. stuttgartiensis* that mapped to the peptidoglycan biosynthesis pathway were then retrieved and their sequences were used as queries in two independent BLAST searches using *B. fulgida* and *S. profunda* protein sequences as reference data sets, respectively. The protein sequences of *B. fulgida* and *S. profunda* which had the best hits with *K. stuttgartiensis* were collected and used as queries in a new BLAST search using the sequences in the NCBI protein database (nr) as the reference data set.

**Batch incubations and activity tests.** The short-term inhibitory effects of lysozyme (lysozyme from chicken egg white; Sigma-Aldrich), penicillin G (penicillin G potassium salt; Sigma-Aldrich), and streptomycin (streptomycin sulfate salt; Sigma-Aldrich) were tested in batch incubations with previously described *Kuenenia stuttgartiensis* free-living planktonic cells (1, 30). For determining the effect of lysozyme, 10 ml cells was incubated with 0.25 to 1 g/liter of lysozyme and with 1 to 20 mM EDTA (the concentrations of lysozyme and EDTA in each incubation are listed in Table S1 in the supplemental material) for 60 min at 37°C (35, 36). If they were not completely lysed after incubation, the lysozyme-treated cells were pelleted by centrifugation for 5 min at 1,200 × g. Then, the pellet was washed with 10 ml of synthetic medium (37) without substrates 3 times or until it was free of EDTA and lysozyme. The cells were resuspended in 10 ml synthetic medium containing 2 mM ammonium and nitrite, followed by anammox activity tests, as previously described (38), with modifications. In short, 10 ml cells was transferred to a 30-ml serum bottle after the pH was adjusted to 7.3. The bottle was sealed with a butyl rubber stopper and an aluminum crimp cap and then repeatedly vacuumed and flushed with Ar-CO2 (95%/5%) to achieve anaerobic conditions before incubation in a shaking incubator (250 rpm) at 30°C. Liquid samples (0.5 ml) were taken every 30 min for ammonium and nitrite measurements until all nitrite was consumed.

For penicillin G and streptomycin, the agents were added to 10 ml of cells and activity tests were performed immediately. Four different concentrations of penicillin G (0.5, 1, 1.5, and 2 g/liter) and streptomycin (30, 100, 150, and 200 mg/liter) were tested.

**Continuous culturing.** The batch experiments were followed by experiments in five successively operated 2-liter (working volume) continuous membrane reactors that were carefully monitored for growth, activity, and cell viability. Each reactor was inoculated with 1 liter of free-living *K. stuttgartiensis* cells and supplied with 500 ml/day of synthetic medium (37) containing 45 mM ammonium and nitrite as the influent. The reactors were flushed continuously with Ar-CO2 (95%/5%); 10 ml/min) to maintain anaerobic conditions. The temperature and pH were maintained at 30°C and 7.3, respectively, with a water bath and bicarbonate solution, respectively. Before the inhibitors were introduced to the reactor, the optical density at 600 nm (OD600) of the biomass was maintained at 0.6 to 0.7, which represented the steady state of the culture, with a constant washout of cells (120 ml/day) (Fig. 1). On day 13, the inhibitor was added to the influent and directly to all reactors except the control reactor to achieve the same concentration as the influent. The control reactor was operated for 60 days without adding any inhibitor (negative control). One of the reactors was inhibited by streptomycin (streptomycin sulfate salt; Sigma-Aldrich) at the working concentration of 100 mg/liter (positive control). This was followed by the operation of three reactors containing different concentrations (0.5, 1, and 5 g/liter) of penicillin G. After the reactors treated with penicillin G became inactive, the cell wash-
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TABLE 1 Genes detected in the genome of *Kuenenia stuttgartiensis* predicted to be involved in peptidoglycan biosynthesis and their transcription levels under two different growth conditions

<table>
<thead>
<tr>
<th>Open reading frame</th>
<th>Strand</th>
<th>Gene</th>
<th>Description</th>
<th>Expression value (RPKM&lt;sup&gt;a&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>kusti1895</td>
<td>R</td>
<td><em>pba</em></td>
<td>Penicillin-binding protein 2</td>
<td>1.38, 2.15</td>
</tr>
<tr>
<td>kusti2372</td>
<td>R</td>
<td><em>queA</em></td>
<td>S-Adenosylmethionine-tRNA ribosyltransferase-isomerase</td>
<td>1.69, 1.88</td>
</tr>
<tr>
<td>kusti2376</td>
<td>F</td>
<td><em>ftsI</em></td>
<td>Division-specific transpeptidase, penicillin-binding protein 3</td>
<td>6.07, 8.85</td>
</tr>
<tr>
<td>kusti2378</td>
<td>F</td>
<td><em>murE</em></td>
<td>UDP-N-acetylmuramyl tripeptide synthase</td>
<td>7.26, 8.46</td>
</tr>
<tr>
<td>kusti2379</td>
<td>F</td>
<td><em>murF</em></td>
<td>UDP-N-acetylmuramoylalanine-α-glutamyl-2,6-diaminopimelate-α-alanyl-α-alanine ligase</td>
<td>6.97, 12.34</td>
</tr>
<tr>
<td>kusti2380</td>
<td>F</td>
<td><em>murY</em></td>
<td>Phospho-N-acetylmuramoyl-pentapeptide transerase</td>
<td>2.48, 3.85</td>
</tr>
<tr>
<td>kusti2383</td>
<td>F</td>
<td><em>murG</em></td>
<td>Undecaprenylphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase</td>
<td>10.44, 12.28</td>
</tr>
<tr>
<td>kusti2385</td>
<td>F</td>
<td><em>murC</em></td>
<td>UDP-N-acetylmuramate-α-alanine ligase</td>
<td>5.53, 6.46</td>
</tr>
<tr>
<td>kusti2386</td>
<td>F</td>
<td><em>dliA</em></td>
<td>d-Alanine-α-alanine ligase</td>
<td>3.72, 10.31</td>
</tr>
<tr>
<td>kusti3293</td>
<td>F</td>
<td><em>mvrN</em></td>
<td>Putative virulence factor, flippase</td>
<td>1.15, 1.21</td>
</tr>
<tr>
<td>kusti3313</td>
<td>R</td>
<td><em>murA</em></td>
<td>UDP-N-acetylglycosamine 1-carboxyvinyltransferase (enolpyruvyl transferase)</td>
<td>5.9, 9.54</td>
</tr>
<tr>
<td>kusti3480</td>
<td>F</td>
<td><em>murD</em></td>
<td>UDP-N-acetylmuramoylalanine-α-glutamate ligase</td>
<td>2.8, 4.34</td>
</tr>
<tr>
<td>kusti3636</td>
<td>R</td>
<td><em>dacB</em></td>
<td>d-Alanyl-α-alanine carboxypeptidase (penicillin-binding protein 4)</td>
<td>1.25, 1.62</td>
</tr>
</tbody>
</table>

<sup>a</sup> R, reverse; F, forward.

<sup>b</sup> RPKM, number of reads per kilobase of exon model per million mapped reads.

RESULTS AND DISCUSSION

Among the 19 *K. stuttgartiensis* genes that were predicted to be involved in peptidoglycan biosynthesis, 13 (Table 1) were suggested to be indispensably required, as determined by comparing the genomic data to data on the genes required for the peptidoglycan biosynthesis pathway using KAAS pathway mapping. The genomes of the other two anammox organisms, freshwater species *B. fulgida* and marine species *S. profunda*, also encode all of the genes indicated by KAAS mapping to be required for peptidoglycan synthesis. For *S. profunda*, all genes essential for peptidoglycan biosynthesis had the highest sequence identity with *K. stuttgartiensis* or anammox species KSU-1 genes, suggested by a BLAST search using the sequences in the NCBI protein database (nr) as a reference data set. For *B. fulgida*, however, 3 genes (the UDP-N-acetylmuramoylalanine-α-glutamate ligase, pentapeptide-transerase, and d-alanyl-α-alanine ligase genes) did not have the best BLAST hits with anammox bacteria (32). The analyses of the other available anammox genomes suggested that other anammox species, as well as *K. stuttgartiensis*, also had the genetic capacity to synthesize a peptidoglycan-like polymer.

When *K. stuttgartiensis* cells were incubated with penicillin G and streptomycin in short-term activity tests, these compounds had no effect on the activity (Fig. 2), which was in line with the previous observations (25). This is probably due to the fact that these compounds are growth inhibitors and the long doubling time of the anammox bacteria makes it impossible to determine their inhibitory effect in 2 to 3 h of short-term batch incubations. Furthermore, when 1 mM EDTA was used with 1 g/liter lysozyme, anammox cells were not lysed and there was no effect on anammox activity. However, when 20 mM EDTA was used with 1 g/liter lysozyme, complete lysis occurred in 60 min. Lysozyme is a very specific glycoside hydrolase that breaks down the 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-β-glucosamine res-
sozyme, is necessary (36). Divalent cations are essential for normal
the outer membranes and exposes the peptidoglycan layer to ly-

concentration of EDTA is necessary to break down both the unique S
layer to the outermost membrane requires bivalent cations and
protein outside the peptidoglycan layer (48). Therefore, for the lysis
Gram-negative bacteria, EDTA, which chelates divalent cations in
the outer membranes and exposes the peptidoglycan layer to ly-
sosome, is necessary. Divalent cations are essential for normal
interactions of cell wall components and in some cases also for
the association of the surface protein or glycoprotein layer (S layer)
with the outer membrane. An S layer is a 2-dimensional layer
composed of identical proteins or glycoproteins that cover an entire
bacterial (Gram positive or Gram negative) or archaeal cell by attaching
to the outermost cell wall membrane. The attachment of
the S layer to the outermost membrane requires bivalent cations and
could be disturbed by the presence of EDTA. The optimal EDTA
centration range for Gram-negative bacterial cell lysis is 0.5 to 2
mM. In our experiments, however, lysis occurred only in the presence of a higher concentration of EDTA, suggesting that in
the anammox bacterial cell wall the peptidoglycan-like structure might
be covered with an additional layer(s) that could be similar to that in
Gram-negative bacteria. Furthermore, a recent study showed that
K. stuttgartiensis has an S layer as the outermost layer of the cell (67). On
the basis of these observations, it could be conceivable that a higher
concentration of EDTA is necessary to break down both the unique S
layer and the outermost membrane of K. stuttgartiensis.

To further assess the inhibitory effects of penicillin G and streptomycin, five continuous membrane reactors were operated
as described above. The control reactor (i.e., the experiment with
out inhibitors) was operated for 60 days without any loss of activity or growth. Streptomycin, which was used as the positive
control, resulted in the washout of anammox bacteria (Fig. 1) and
nitrite accumulation. In the last 3 days of operation, the concen-
tration of nitrite in the effluent increased from 0 to over 5 mM. The
bio reactor was completely inactivated within 17 days after the
introduction of streptomycin into the reactor, which corresponds
to the washout rate of the reactor (16.6 days).

Interestingly, at all tested concentrations, penicillin G (0.5, 1,
and 5 g/liter) also inhibited the growth of anammox bacteria, as
determined by the OD600 and deactivated the reactor completely.
This was observed as a rapid nitrite accumulation after approxi-
mately 3 weeks of treatment, similar to the effect of streptomycin (Fig. 1). These results show that the anammox bacteria are sensitive
to β-lactam antibiotics and, together with the results of the batch incubations with lysozyme, suggest that the cell walls of the
anammox bacteria contain peptidoglycan-like polymers and PBPs
are involved in their synthesis.

Furthermore, when the biomass from the penicillin G-inhib-
ited bioreactor was inspected with phase-contrast and epifluores-
cence microscopy (after fluorescence in situ hybridization and
dNA staining with DAPI), bloated anammox cells (approximately twice the size of normal cells) were observed (Fig. 3), indicat-
ing that K. stuttgartiensis is unable to grow or divide properly and
undergoes plasmolysis. One of the genes in the K. stuttgartiensis
genome encodes cell division protein FtsI (Kuste2376). This protein
is also known as PBP 3 and is involved in septal peptidoglycan synthesis during cell division (55, 56). It is conceivable
that the case of K. stuttgartiensis, penicillin also bound PBP 3 and inhibited cell division of the anammox bacteria.

When penicillin G addition was stopped and the reactor was
supplied with mineral medium (37) without nitrite, the accumu-
lated nitrite was completely removed within 3 to 4 days. Once
nitrite was below the detection limit (<50 μM), ammonium and
nitrate (45 mM each) were supplied once more. This resulted in an
increase of the optical density (OD600), indicating the anammox
bacteria were growing again (Fig. 1). Apparently, protein synthesis
had not stopped completely in the penicillin-inhibited cells, and
these were most likely able to synthesize new PBPs. During this
period (between days 45 and 60), the doubling times of the resus-
citated reactors treated with 0.5, 1, and 5 g/liter penicillin G were
calculated to be 14, 17, and 18 days, respectively. These doubling
times were longer than the doubling time of the control reactor
under steady-state growth, which was calculated to be ~11 days
(57).

We further investigated the effect of penicillin on the anam-
nox bacteria by sequencing the transcriptome of K. stuttgartiensis
from the control reactor and from the reactor that was treated
with 0.5 g/liter penicillin G. When RNA quality was examined, it
was revealed that RNA quantity dropped significantly (over 10-
fold; data not shown) after 3 weeks of penicillin G treatment,
suggesting that the protein synthesis machinery of the cell was
turned down. In total, in the penicillin G-treated sample, 1,759
genes were neither up- nor downregulated, 258 were significantly
downregulated (over 2-fold), and 37 of these were downregulated
over 5-fold (see Table S2 in the supplemental material).

The transcriptome analysis revealed that, in line with the observa-
tion that cells stopped dividing, the anammox-specific cell division
protein Kustd1438, which was reported to be a replacement of the
protein for the cell division gene encoding the tubulin analogue FtsZ
(54), was downregulated 25-fold. All genes involved in peptidoglycan
biosynthesis encoded by K. stuttgartiensis were expressed in both
samples. However, none of them was significantly up- or downregu-
lated. Furthermore, miRNA transcribed from the gene (kustu0010)
encoding a membrane-bound lytic transglycosylase-like protein was detected in both samples. The function of this protein was suggested to be to remodel the peptidoglycan layer during cell enlargement and division by catalyzing the cleavage of the β-1,4-glycosidic bond in peptidoglycan that is necessary for the insertion of new monomers (58, 59). Besides, lytic transglycosylases could also bind to many types of PBPs (bifunctional and monofunctional) to form a complex that has been indicated to be involved in peptidoglycan biosynthesis (59–61).

The ultimate result of the penicillin inhibition in the continuous cultures was the accumulation of nitrite in the effluent of the reactor. Interestingly, one of the genes encoding a putative nitrite transporter (Kuste3055) was downregulated 15-fold. It was previously reported that this gene is by far the most expressed among the genes for nitrite transporters in \textit{K. stuttgartiensis} (1, 62). Apparently, the response of the cell to elevated nitrite concentrations was to shut down nitrite import into the cell. In \textit{K. stuttgartiensis}, nitrite is reduced to nitric oxide (NO) by nitrite reductase NirS (Kuste4136) (62). Surprisingly, in the penicillin G-treated sample, Kuste4136 was downregulated 63-fold. Apparently, the cells responded to high nitrite concentrations not by upregulating the nitrite-converting enzyme but by shutting down their catabolic machinery. Interestingly, this observed response was similar to the response of a nitrite reductase (NirK)-deficient mutant of the aerobic ammonium oxidizer \textit{Nitrosomonas europaea}, which, in response to nitrite toxicity, downregulated its nitrite detoxification genes (63).

In contrast to the downregulation of 258 genes, only 47 genes were significantly upregulated (over 2-fold), and 9 of them were upregulated over 5-fold (see Table S3 in the supplemental material). The most upregulated functional gene was \textit{kustd1340} (62), which encodes the second copy of Kustc0694. The product of Kustc0694 was previously purified and identified to be hydrazine dehydrogenase (HDH), which is responsible for the four-electron oxidation of hydrazine to \(N_2\) (1). The transcription levels of Kustc0694 under both growth conditions were high (and it was among the transcripts with the highest levels of transcription detected, with an RPKM value of 3,707 in the penicillin G-treated sample and 4,109 in the control sample) but without significant up- or downregulation. Until now, the physiological conditions where \textit{kustd1340} would be expressed have been unknown. Our results indicated that the \textit{kustd1340} gene was at least transcribed to mRNA under extreme cellular stress. This indicated that the second copy of HDH could serve as a backup system under stress conditions. Future research will be aimed at better defining these stress conditions and the purification of the \textit{kustd1340} gene product.
Both antibacterial agents used in this study that target peptidoglycan-like polymers and PBPs, lysozyme and penicillin G, were detrimental for the anammox bacteria. These results indicate that a polymer that could be lysed by lysozyme and the synthesis of which could be inhibited by penicillin G is present in the cell wall of the anammox bacteria. It should be noted that neither of the two PBPs encoded by the K. stuttgartiensis genome has predicted transglycosylase activity, which is essential for the elongation of glycan strands of peptidoglycan polymer (16). However, it was reported that the transglycosylase PBP is also missing in C. thomatis. It was hypothesized that instead of normal peptidoglycan, C. thomatis could possess a glycan-less polypeptide on its cell wall (64). Moreover, it was reported that PBP 3, which was considered a monofunctional PBP with transpeptidase activity only, might have transglycosylase activity as well (65). Further, for some cyanobacteria, even though they have transglycosyl PBP3s, in a mutant lacking this kind of PBP, the mutation was not lethal (66). All aforementioned cases indicated that the transglycosylase PBP3s might not be absolutely necessary for the biosynthesis of peptidoglycan or a peptidoglycan-like component that does not have a glycan backbone. Any of these cases could be true for the anammox bacteria and need further dedicated research to be resolved.

In the present study, we showed the short- and long-term effects of three antibacterial agents on anammox bacteria by batch incubations and continuous cultivation. For each reactor operated in this study, it took only 2 weeks after inoculation to reach an apparent steady state and approximately 3 weeks after the introduction of inhibitory to the conclusion of the experiments. Our observations show that the continuous membrane reactor has manifold advantages for investigation of the effects of certain compounds on anammox bacteria, such as antibacterial agents in this study. Taking into account the fact that anammox bacteria are notoriously difficult to culture and are slow growing, the availability of a 5-week operation period for bio reactors is a significant step forward in investigating the effects of certain compounds (in this case, inhibitors), and if they are implemented in other laboratories, they could accelerate the research on the physiology of slow-growing microorganisms, in particular, anammox bacteria.

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