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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.

Anaerobic ammonium-oxidizing (anammox) bacteria oxidize ammonium with nitrite as the terminal electron acceptor and with nitric oxide and hydrazine as intermediates (1). These microorganisms contribute significantly to the release of fixed nitrogen back to the atmosphere (2–4) and are applied in wastewater treatment as an environmentally friendly and cost-effective method of nitrogen removal (5).

All known anammox bacteria belong to the phylum *Planctomycetes* (6). Members of this phylum are unique in many aspects; for example, they have a complex cell compartmentalization (7), an unusual fatty acid composition of the phospholipids (8), and the lack of peptidoglycan on their cell wall (9, 10). Peptidoglycan is a major cell wall component present in almost all bacteria (11) but was not detected in planctomycetes with biochemical assays (10, 12). Furthermore, classical peptidoglycan was not observed in anammox bacteria in ultrastructural studies (13, 14). Planctomycetes, together with the chlamydiae and cell-wall-less mycoplasmas, are the only known peptidoglycan-lacking microorganisms within the domain *Bacteria* (7).

A peptidoglycan monomer is composed of a pentapeptide component and a glycan strand consisting of two connected amino sugar residues, *N*-acetylmuramic acid and *N*-acetylglucosamine (15). In bacterial cell walls, peptidoglycan monomers join together by concatenated glycan strands, catalyzed by transglycosylases, and cross-linked short-stem peptides, catalyzed by transpeptidases, to form three-dimensional mesh-like layers that provide bacteria structural integrity and enable them to resist osmotic lysis (16). Penicillin-binding proteins (PBPs) are involved in the final stage of peptidoglycan biosynthesis, the formation of peptidoglycan cross-links, cell separation, and peptidoglycan maturation or recycling of monomers (17, 18).

PBPs that catalyze the cross-linkage of peptidoglycan can be divided into two classes on the basis of their activity: bifunctional PBPs and monofunctional PBPs. Bifunctional PBPs normally have both transglycosylase and transpeptidase activities, and monofunctional PBPs have transpeptidase activity only (16). The disorder of PBPs or the peptidoglycan monomer itself therefore leads to cell lysis and death for both Gram-positive and Gram-negative bacteria (11, 18, 19). There are several compounds, such as lysozyme and β-lactam antibiotics, that attack the integrity of peptidoglycan and as such are used as antibacterial agents. *N*-Acetylmuramide glycan hydrolase (lysozyme) hydrolyzes the glycosidic bonds of the peptidoglycan monomer (20, 21), whereas β-lactam antibiotics, such as penicillin, inhibit the cross-linking of peptidoglycan by binding to PBPs (11). Some bacteria are resistant to penicillin or other types of β-lactam antibiotics because they harbor β-lactamases or have developed special PBPs with a very low affinity for these antibiotics (22, 23).

Theoretically, anammox bacteria and all other planctomycetes which have peptidoglycan-lacking cell walls should not be sensitive to β-lactam antibiotics, including penicillin G (24). Indeed, one of the first papers on the anammox bacteria reported that penicillin (penicillin V) had no inhibitory effect on anammox activity (25). Further, Güven et al. used 0.5 g/liter penicillin G (a concentration 5 times higher than the normal working concentration of penicillin G) to inhibit possible heterotrophic denitrifier

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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 (*kustd1895* and *kuste2376*) encode proteins that are homologous to enzymes that are suggested to be monofunctional (transpeptidase) PBPs (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/M. 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-CO_2 (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.

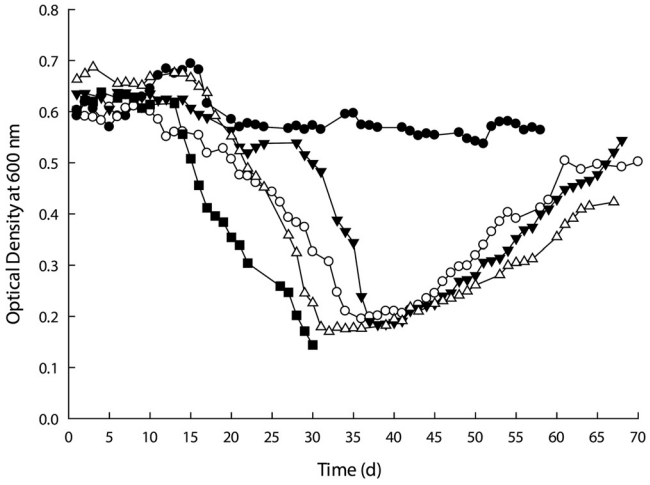

FIG 1 Effects of penicillin G and streptomycin on *Kuenenia stuttgartiensis* single-cell enrichment culture. Filled circles, no inhibitor; open circles, 0.5 g/liter penicillin G; filled triangles, 1 g/liter penicillin G; open triangles, 5 g/liter penicillin G; filled squares, 100 mg/liter streptomycin.

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 (50, 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-CO_2 (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 (OD_{600}) 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-

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

Open reading frame	Strand[a]	Gene	Description	Expression value (RPKM[b])	
				Penicillin G treated	Control
kustd1895	R	*pbpA*	Penicillin-binding protein 2	1.38	2.15
kuste2372	R	*queA*	*S*-Adenosylmethionine-tRNA ribosyltransferase-isomerase	1.69	1.88
kuste2376	F	*ftsI*	Division-specific transpeptidase, penicillin-binding protein 3	6.07	8.85
kuste2378	F	*murE*	UDP-*N*-acetylmuramyl tripeptide synthase	7.26	8.46
kuste2379	F	*murF*	UDP-*N*-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate–D-alanyl–D-alanine ligase	6.97	12.34
kuste2380	F	*mraY*	Phospho-*N*-acetylmuramoyl-pentapeptide transferase	2.48	3.85
kuste2383	F	*murG*	Undecaprenyldiphospho-muramoylpentapeptide beta-*N*-acetylglucosaminyltransferase	10.44	12.28
kuste2385	F	*murC*	UDP-*N*-acetylmuramate–L-alanine ligase	5.53	6.46
kuste2386	F	*ddlA*	D-Alanine:D-alanine ligase	3.72	10.31
kuste3293	F	*mviN*	Putative virulence factor, flippase	1.15	1.21
kuste3313	R	*murA*	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (enolpyruvyl transferase)	5.9	9.54
kuste3480	F	*murD*	UDP-*N*-acetylmuramoylalanine D-glutamate ligase	2.8	4.34
kuste3636	R	*dacB*	D-Alanyl–D-alanine carboxypeptidase (penicillin-binding protein 4)	1.25	1.62

[a] R, reverse; F, forward.
[b] RPKM, number of reads per kilobase of exon model per million mapped reads.

out was stopped, fresh synthetic medium was supplied as the influent until all accumulated nitrite was consumed, and synthetic medium containing 45 mM ammonium and nitrite but no penicillin G was supplied again to resuscitate the reactors.

Analytical methods. Liquid samples from reactors and activity tests were pelleted by centrifugation for 5 min at 16,000 × *g*. The supernatants were transferred to new tubes and stored at −20°C until further analyses. The concentrations of ammonium and nitrite were measured colorimetrically as previously described (39). Protein concentrations were measured using the biuret method, as described previously (40).

FISH and phase-contrast microscopy. One milliliter of liquid sample was taken from the reactor deactivated by penicillin G and the negative-control reactor. Sample fixation and fluorescence *in situ* hybridization (FISH) were performed as described previously (41). Probe AMX820, specific for *Kuenenia*- and *Brocadia*-like anammox bacteria (42), was used to detect *K. stuttgartiensis*, and a mixture of probes EUB1 to EUB4, specific for most bacteria (43–45), was used to visualize most bacteria. DAPI (4′,6-diamidino-2-phenylindole) was used to stain the whole community DNA.

For phase-contrast microscopy, 15 μl of liquid sample was taken from both reactors at the same time point as that at which samples were obtained for FISH analyses and directly observed by use of an Axioplan 2 imaging system (Carl Zeiss, Germany) without fixation.

RNA isolation and transcriptome sequencing and analyses. Transcriptome sequencing was performed on samples from the control reactor and the reactor treated with 0.5 g/liter penicillin G. In short, equal amounts of cells were harvested on day 35 from both reactors, and total RNA was extracted with a RiboPure-Bacteria kit (Ambion) according to the manufacturer's instructions. RNA quality in terms of the amount and size distribution was examined by an Agilent 2100 bioanalyzer (Agilent) before library construction. Sequencing library construction and transcriptome sequencing were performed using an Ion total transcriptome sequencing (RNA-Seq) kit and an Ion PGM 200 sequencing kit (Ion Torrent), respectively.

Mapping of the transcriptome reads was performed with CLC Genomics Workbench software (CLC Bio, Denmark) using the RNA-Seq analysis tool with a minimum length of 95%, a minimum identity of 95%, and the genome of *K. stuttgartiensis* as a reference. All reads that mapped to rRNA and tRNA genes were excluded from the results. The number of reads per kilobase of the exon model per million mapped reads (RPKM) values of all protein-coding sequences (CDS) of penicillin G-treated samples and control samples were retrieved and compared to each other to identify the expression level changes.

Nucleotide sequence accession number. The transcriptome sequences have been deposited in the Sequence Read Archive (SRA) under accession number PRJNA219373 (http://www.ncbi.nlm.nih.gov/bioproject/219373).

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–D-glutamate ligase, pentapeptide-transferase, and D-alanyl–D-alanyl ligase genes) did not have 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-D-glucosamine res-

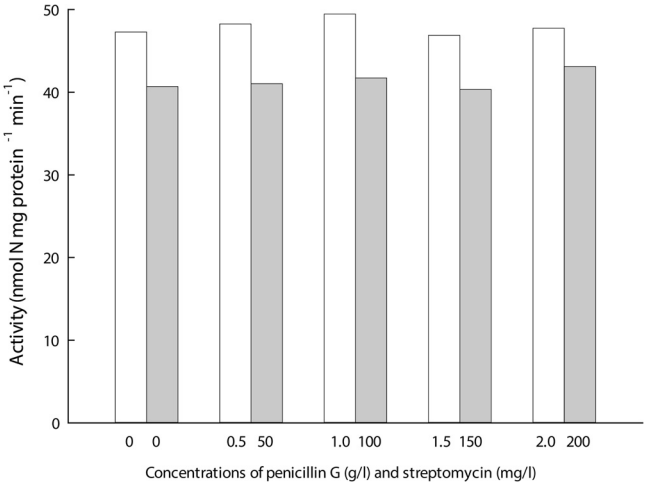

FIG 2 Short-term effects of penicillin G and streptomycin on anammox activity. White bars, biomass treated with penicillin G; gray bars, biomass treated with streptomycin.

idues in a peptidoglycan monomer and between *N*-acetyl-D-glucosamine residues in chitodextrins (46, 47). The complete lysis observed here indicated that the cell wall of the anammox bacteria contained molecules that lysozyme could target, most likely a peptidoglycan-like molecule. Due to this almost immediate lysis effect, continuous cultures were not operated with lysozyme.

Unlike Gram-positive bacteria, in which cross-linked peptidoglycan is in the outermost layer of the cell wall, in Gram-negative bacteria, there is an outer membrane containing lipopolysaccharides and protein outside the peptidoglycan layer (48). Therefore, for the lysis of Gram-negative bacteria, EDTA, which chelates divalent cations in the outer membranes and exposes the peptidoglycan layer to lysozyme, is necessary (36). 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 (49, 50). 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 (51, 52). The attachment of the S layer to the outermost membrane requires bivalent cations and could be disturbed by the presence of EDTA (51). The optimal EDTA concentration range for Gram-negative bacterial cell lysis is 0.5 to 2 mM (53). 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 without 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 concentration of nitrite in the effluent increased from 0 to over 5 mM. The bioreactor 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 OD_{600}, and deactivated the reactor completely. This was observed as a rapid nitrite accumulation after approximately 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-inhibited bioreactor was inspected with phase-contrast and epifluorescence 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), indicating 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) (27, 54). This protein is also known as PBP 3 and is involved in septal peptidoglycan synthesis during cell division (55, 56). It is conceivable that in 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 accumulated nitrite was completely removed within 3 to 4 days. Once nitrite was below the detection limit (<50 μM), ammonium and nitrite (45 mM each) were supplied once more. This resulted in an increase of the optical density (OD_{600}), 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 resuscitated 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 anammox 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 rRNA 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 observation 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 downregulated. Furthermore, mRNA transcribed from the gene (*kusta0010*)

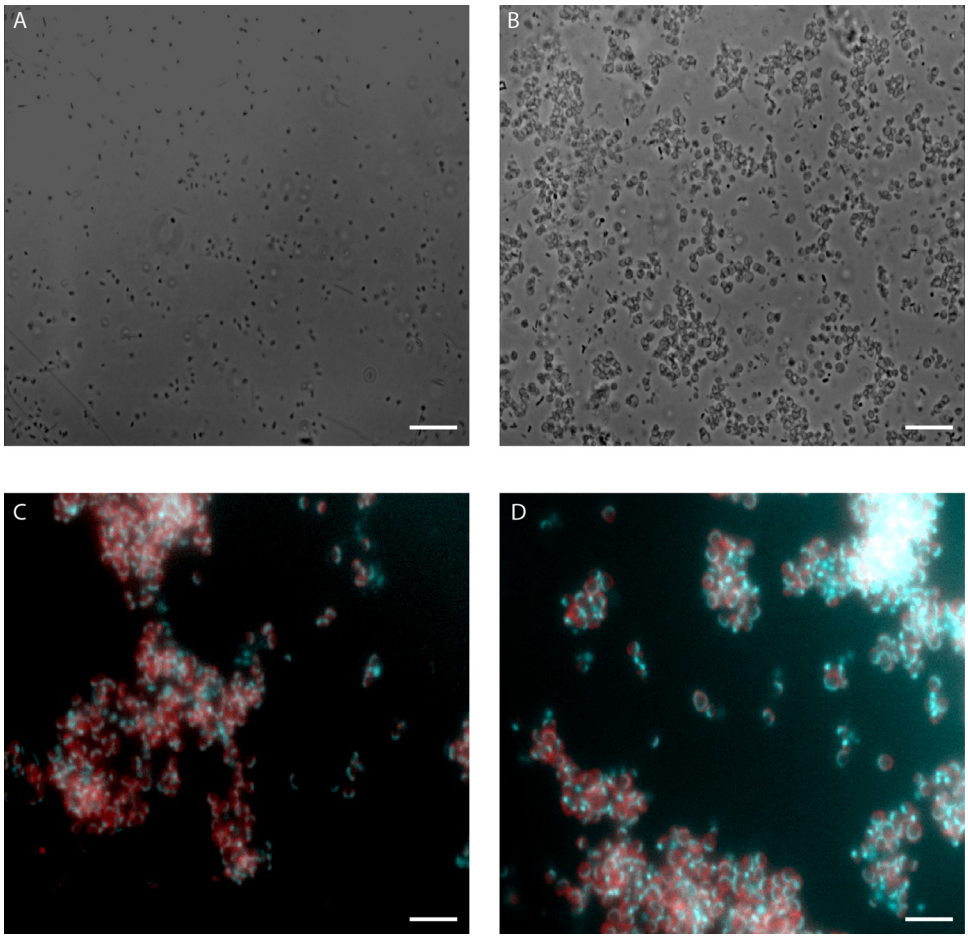

FIG 3 Phase-contrast (A, B) and FISH (C, D) micrographs of *Kuenenia stuttgartiensis* single cells before (A, C) and after (B, D) 3 weeks of 1-g/liter penicillin G treatment. The AMX820 probe targeting *Brocadia*- and *Kuenenia*-like anammox bacteria was labeled with Cy3 (red). Staining with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; light blue fluorescence) targeting double-stranded DNA is also shown. Bars = 5 μm.

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 *K. stuttgartiensis* (1, 62). Apparently, the response of the cell to elevated nitrite concentrations was to shut down nitrite import into the cell. In *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 *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 *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 Kustd1340 would be expressed have been unknown. Our results indicated that the *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 *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 *Chlamydia trachomatis*. It was hypothesized that instead of normal peptidoglycan, *C. trachomatis* 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 transglycosylase PBPs, in a mutant lacking this kind of PBP, the mutation was not lethal (66). All aforementioned cases indicated that the transglycosylase PBPs 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 inhibitors 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 bioreactors 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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