Effects of selective digestive decontamination (SDD) on the gut resistome

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Objectives: Selective digestive decontamination (SDD) is an infection prevention measure for critically ill patients in intensive care units (ICUs) that aims to eradicate opportunistic pathogens from the oropharynx and intestines, while sparing the anaerobic flora, by the application of non-absorbable antibiotics. Selection for antibiotic-resistant bacteria is still a major concern for SDD. We therefore studied the impact of SDD on the reservoir of antibiotic resistance genes (i.e. the resistome) by culture-independent approaches.

Methods: We evaluated the impact of SDD on the gut microbiota and resistome in a single ICU patient during and after an ICU stay by several metagenomic approaches. We also determined by quantitative PCR the relative abundance of two common aminoglycoside resistance genes in longitudinally collected samples from 12 additional ICU patients who received SDD.

Results: The patient microbiota was highly dynamic during the hospital stay. The abundance of antibiotic resistance genes more than doubled during SDD use, mainly due to a 6.7-fold increase in aminoglycoside resistance genes, in particular \( \text{aph}(2')^{-}\text{Ib} \) and an \( \text{aadE} \)-like gene. We show that \( \text{aph}(2')^{-}\text{Ib} \) is harboured by anaerobic gut commensals and is associated with mobile genetic elements. In longitudinal samples of 12 ICU patients, the dynamics of these two genes ranged from a \( \approx 10^4 \) fold increase to a \( \approx 10^2 \) fold decrease in relative abundance during SDD.

Conclusions: ICU hospitalization and the simultaneous application of SDD has large, but highly individualized, effects on the gut resistome of ICU patients. Selection for transferable antibiotic resistance genes in anaerobic commensal bacteria could impact the risk of transfer of antibiotic resistance genes to opportunistic pathogens.

Keywords: intensive care medicine, antibiotic resistance, metagenomics

Introduction

Infections are a major threat to hospitalized patients, especially to those treated in intensive care units (ICUs), where infections are associated with increased morbidity, mortality and healthcare costs.1,2 Selective decontamination of the digestive tract (SDD) is an infection prevention measure that reduces ICU-acquired respiratory tract infections and bacteraemia and improves the survival of ICU patients,3,4 through the eradication of potential pathogenic microbes in the oropharynx and the digestive tract, while leaving the anaerobic microbiota undisturbed.5 SDD involves the administration of non-absorbable antibiotics (colistin and tobramycin) and an antifungal (amphotericin B) into the oropharynx and intestinal tract during the ICU stay, in combination with the intravenous administration of a third-generation cephalosporin (usually cefotaxime) during the first 4 days in ICU. Despite the reported benefits of SDD, this intervention is currently not widely used, primarily because of concerns that it may select for antibiotic-resistant bacteria in the patient’s microbiota.5 However, a recent meta-analysis of 64 clinical studies failed to demonstrate that SDD increased the number of infections caused by antibiotic-resistant pathogens.7 An important limitation of all studies included in this meta-analysis is that they relied on conventional culture techniques, which are unable
to capture anaerobic commensals, such as Clostridia and Bacteroidetes. Anaerobic bacteria constitute the majority of the gut microbiota and can carry a large reservoir of antibiotic resistance genes, i.e. the resistome. Antibiotics may select for antibiotic resistance genes carried by gut commensal bacteria and thereby facilitate horizontal gene transfer to opportunistic pathogens. Consequently, to fully evaluate the safety of SDD in ICU settings, its effect on the patient gut resistome needs to be assessed.

Here, we describe the dynamics of the gut microbiota and the resistome in detail in a single patient admitted to ICU after a traffic accident and who received SDD for 17 days. Samples were taken at Days 4, 14 and 16 in ICU, at Day 28 (during post-ICU hospitalization) and at Day 313 (270 days after hospital discharge). We subsequently studied the dynamics of two anaerobic bacteria in the gut microbiota of 12 ICU patients who received SDD. Our data indicate that SDD can have large, but highly individualized, effects on the patient resistome.

Methods

Patient information

The patient who was the main subject of this study had no previous history of hospitalization and disease. Upon ICU admission, the patient presented with an acute neurological trauma due to a basal skull fracture after a traffic accident. Additional screening for trauma showed no abnormalities. Microbiological surveillance of the patient was performed according to conventional culturing techniques on an almost daily basis. Rectal cultures were grown on blood agar, MacConkey agar and malt agar. Sputum cultures were grown on blood agar containing aprotinin, MacConkey agar, malt agar and Haemophilus chocolate agar. Blood samples were monitored in a BD BACTEC FX machine according to standard laboratory practice. Culture-based diagnostics failed to detect any pathogenic, antibiotic-resistant bacteria at any timepoint in any sample (Table S1, available as Supplementary data at JAC Online).

The patient received SDD, with 1000 mg of cefotaxime intravenously four times daily for 4 days and an oropharyngeal paste containing polyvidone tobramycin and amphotericin B (each at a concentration of 2%) and administration of a 10 ml suspension containing 100 mg of polymyxin E, 80 mg of tobramycin and 500 mg of amphotericin B via a nasogastric tube, four to eight times daily throughout the ICU stay. Additional information on the antibiotic therapy that the patient received throughout the study period is provided in Table S2 (available as Supplementary data at JAC Online).

Strains and growth conditions

Escherichia coli EP1300-T1R (Epitope, Madison, WI, USA) was used for fosmid library construction (further described below) and E. coli TOP10 (Invitrogen, Life Technologies Europe BV, Netherlands) for other genetic manipulations. E. coli was grown in Luria broth (LB; Oxoid) at 37°C. Antibiotics were used at the following concentrations: chloramphenicol, 12.5 mg/L; ampicillin, 100 mg/L; tobramycin, 25 mg/L; tetracycline, 10 mg/L; erythromycin, 500 mg/L; colistin, 50 and 10 mg/L; cefotaxime, 25 mg/L; and cefazolin, 32 mg/L.

Faeces collection and isolation of high molecular weight DNA

Faeces from the patient described above were collected upon defecation and stored at 4°C between 30 min and 4 h, after which the faeces were transferred to –80°C. For DNA isolation, an aliquot of ~15 g of faecal matter was defrosted and homogenized in PBS (138 mM NaCl/2.7 mM KCl/140 mM Na2HPO4/1.8 mM KH2PO4, adjusted to pH 7.4 with HCl) by vigorous vortexing and layered on a Nycodenz AG gradient (Axis-Shield PoC, Oslo, Norway). The cellular fraction of the faecal matter was then separated via centrifugation at 16000 g for 6 min. After removal of the upper layer, the bacterial cellular fraction was recovered and washed three times in PBS, as previously described.

High molecular weight DNA was extracted from the cell pellet as previously described, with minor modifications. Briefly, the recovered cells were lysed with lysozyme (10 mg/ml; Sigma Aldrich, St Louis, MO, USA) and mutanolysin (100 U/ml; Sigma Aldrich), followed by protease K (50 mg/ml; Sigma Aldrich) digestion and addition of 2.5% n-lauryl sarcosine (Sarkosyl; Sigma Aldrich). Proteins were precipitated with 10 M ammonium acetate, and DNA was extracted with chloroform using phase-lock tubes (5 Prime, Gaithersburg, MD, USA) and ethanol precipitation. The quantity and purity of DNA were measured using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE, USA).

Phylogenetic profiling of the gut microbiota

The faecal DNA isolated above was used to phylogenetically profile the gut microbiota using HITChip analysis, as described previously.

Metagenomic shotgun sequencing and sequence analysis

DNA library construction and sequencing was performed by BGI (Shenzhen, China) using 91 nt paired-end sequencing on an Illumina HiSeq 2000 system as described elsewhere. Between 125 and 221 million high-quality reads were generated for the five samples.

Using SOAPdenovo (http://soap.genomics.org.cn), sequence data were assembled into contigs larger than 500 nt for which between 78.6% and 89.0% of the reads could be used in the assemblies. Further details on the results of the metagenomic shotgun sequencing and de novo assembly are provided in Table S3 (available as Supplementary data at JAC Online).

We used BLAST to detect the presence of antibiotic resistance genes in the different assemblies of each sample. We initially extracted a set of antibiotic resistance gene sequences from the Resistance Determinants Database (RED-DB; http://www.fibim.unisi.it/REDDB). To reduce the redundancy in this database, we first clustered the nucleotide sequences using CD-HIT with a threshold of 99% identity. The clustered resistance gene database was used as a query in a local BLAST search on each assembled sample. All hits with a nucleotide identity of 90% or higher and covering >50% of the query length were considered to be resistance genes that were encoded on the assembled contigs. Relative quantification of the resistance genes per sample was performed as follows. First, the average sequencing depth over the entire assembly was calculated, and then the coverage of the individual contigs, determined using soap.coverage (http://soap.genomics.org.cn), encoding resistance genes was divided by the average sequencing depth over the entire assembly. Data were then log2-transformed and plotted onto a heatmap using MultiExperiment Viewer (http://www.tmm4.org/mev/). Non-transformed abundance data for each resistance gene are provided in Table S4 (available as Supplementary data at JAC Online).

Construction of fosmid libraries

The construction of fosmid libraries was performed using the CopyControl Fosmid Library Production Kit (Epitope) according to the manufacturer’s instructions with slight modifications. Size selection of ~40 kb DNA fragments was performed using PFGE using the CHEF-DR II system (Bio-Rad) with the following settings: initial switch time, 0.1 s; final switch time, 10 s; 4 V/cm; and running time 17 h. The DNA was excised from the gel at the height of a 40 kb marker (Fosmid Control DNA, Epitope), recovered using.
GEIse (Epigen) and end-repaired using the End-It kit (Epigen). DNA was then purified using SureCLean (Bioline, London, UK), and used for ligation. Packaged phage extracts were diluted in 0.5 mL of phage dilution buffer and added to 5 mL of phage-resistant EP1300-T1 E. coli for 1 h at 37 °C. Serial dilutions of the transduced E. coli were plated onto LB agar plates containing chloramphenicol. Libraries were harvested by scraping the plates and suspending the colonies in LB containing 20% glycerol and chloramphenicol, and were frozen in liquid nitrogen and stored at −80°C.

### Identification and characterization of antibiotic-resistant clones in fosmid libraries

The fosmid libraries were plated in 1-, 10-, and 100-fold dilutions on LB agar with chloramphenicol supplemented with tobramycin, ampicillin, tetracycline, erythromycin, cefotaxime, colistin, and cefazolin and incubated at 37 °C overnight. A vector-only control (E. coli EP1300-T1′ with the fosmid library vector pCC1FOS) was also included and only produced colonies on plates supplemented with chloramphenicol but not on plates containing chloramphenicol and the other antibiotics. Quantification of resistant clones was performed in duplicate by plating serial dilutions of the libraries on LB agar supplemented with chloramphenicol in addition to the antibiotic of interest. The total number of clones in the library was determined by plating on LB with chloramphenicol only.

To ensure that resistant clones were due to the fosmid insert and not to spontaneously occurring mutations, five clones per library and per antibiotic were randomly selected from plates and were restreaked onto LB plates containing the appropriate antibiotics. After overnight growth of the restreaked clones, clones were picked and subsequently cultured in LB broth containing the appropriate antibiotics for fosmid isolation. Fosmids were induced to high-copy by the CopyControl Fosmid Autoinduction Solution from Epigen prior to fosmid isolation to increase the total DNA yield. Fosmids were purified using the Qiagen (Venlo, The Netherlands) Miniprep kit. The elution buffer was heated to 70°C prior to elution of the fosmids from the column. The isolated fosmids were then used to transform chemically competent EP1300-T1′ E. coli by heat shock. The transformed clones were restreaked on LB agar with chloramphenicol in addition to the antibiotics used for the initial resistance screening. A clone of EP1300-T1′ E. coli freshly transformed with the fosmid vector pCC1FOS was used as a control throughout. All phenotypes for the selected clones were confirmed, while E. coli with pCC1FOS remained unable to grow.

To assess fosmid insert diversity, fosmids of the selected clones were digested with MsI (New England Biolabs, Ipswich, MA, USA). Differences in restriction patterns were used as indicators for the diversity among the isolated clones. The most prominent clones were subsequently selected for transposon mutagenesis to functionally identify the resistance determinants.

In order to identify the resistance genes on the fosmids that were responsible for causing the resistant phenotype in E. coli, transposon mutagenesis was performed using the EZ-Tn5 -KAN-2-<KAN-2> and EZ-Tn5 -TET-1< in vitro transposon mutagenesis kits (Epigen). Transposon mutagenesis was carried out according to the manufacturer’s instructions with the exception that 5 mM MgCl2 was added to the LB agar when using the EZ-Tn5 -KAN-2-<KAN-2> kit. After in vitro transposon mutagenesis, between 100 and 300 transposon mutants were streaked onto LB agar with chloramphenicol and LB agar with chloramphenicol and ampicillin, tobramycin or tetracycline to screen for the loss of the resistant phenotype. For each in vitro transposon mutagenesis, between one and five clones could be identified that lost their resistance phenotype due to transposon insertion. Sequencing primers TET-1 FP-1 Forward Primer, TET-1 RP-1 Reverse Primer, KAN-2 FP-1 Forward Primer and KAN-2 RP-1 Reverse Primer (provided by Epicentre) were used to sequence along the transposon insertion sites and thereby identify the resistance genes.

Partial sequences from the inserts cloned into the different fosmid and transposon insertion sites were obtained by standard Sanger sequencing. To identify the resistance genes based on the transposon insertion site sequences, we used the RED-DB (http://www.fibim.unisi.it/REDDB/). For each resistant phenotype, several clones that had lost their resistant phenotype upon transposon insertions were analysed. After analysis of the transposon insertion site sequences, we identified the same resistance determinant per antibiotic-resistant clone (tobramycin, ampicillin, and erythromycin) and therefore only chose one fosmid clone per antibiotic to be fully sequenced subsequently. We identified two different tetracycline resistance determinants, and two representative fosmids were selected for sequencing.

Fosmids were pooled and sequenced via Illumina sequencing on a HiSeq 2000 system using the Illumina CASAVA pipeline version 1.8.2 generating paired end reads (read length 101 bp) with an average insert size between 265 bp and 384 bp. Assembly was performed using the CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). The DNA sequences of the regions where the foecal DNA was cloned into the fosmid backbone and of the resistance gene previously obtained by Sanger sequencing were also used to assemble the fosmid insert. Finally, fosmid insertion sequences (ISSs) were closed by sequencing PCR products that spanned the gaps between the contigs in the assembly of each ISS.

The taxonomic classification and identification of the putative source organism of fosmid ISSs was performed using WebCARMA. Annotation of the fosmid ISSs was generated using the prokaryotic annotation pipeline offered by Integrative Services for Genomic Analysis. Annotations were visualized using the Geneious software package (http://www.geneious.com). The ACLAME server was used to identify and classify putative mobile genetic elements within the fosmid sequences. IS elements were identified using ISfinder.

### Quantification of aph(2′)-Ib and the aadE-like gene in ICU patient microbiota by quantitative PCR (qPCR)

To further determine the effect of the ICU hospitalization and SDD on the relative abundance of aph(2′)-Ib and the aadE-like aminoglycoside resistance gene in the gut microbiota of patients, faecal samples were collected from 12 patients that were hospitalized in the ICU for 9 days or longer. Two or three faecal samples per patient were collected during their ICU stay.

DNA was isolated from 200 mg stool samples using the repeated mechanical bead beating method combined with the QIAamp DNA stool MiniKit (Qiagen) as described elsewhere. The DNA samples were used in qPCRs to quantify the copy number of aph(2′)-Ib and the aadE-like gene. All qPCRs were carried out in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems), sealed with optical adhesive film (Applied Biosystems), and using a StepOnePlus™ Real-Time PCR cycler (Applied Biosystems) with StepOnePlus software version 2.2 (Applied Biosystems). The total reaction volume was 25 μL using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) according to the manufacturer’s instructions with a primer concentration of 200 nM and 1 ng of DNA. Primers were designed for the targeted resistance genes aph(2′)-Ib (forward primer: 5′-GGATTGCTCAGGATTCA-3′) and the aadE-like gene (forward primer: 5′-CCATTTGCTGTGCGTATT-3′; reverse primer: 5′-CCATTTGCTGTGCGTATT-3′) using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). The universal primers for 16S rDNA genes were previously described by Gloor et al. and PCR conditions were previously described by van den Bogert et al. Melting curves were included for each qPCR run. The relative abundance of the resistance genes was calculated using the 2−ΔΔCT method with 16S rDNA as the universal housekeeping gene. The relative abundance of the resistance genes in the first faecal sample that was collected during ICU hospitalization was normalized to 1, and subsequent samples were compared with this first sample. The qPCRs were performed with three technical replicates.
Statement of ethical approval

The protocol for this study was reviewed and approved by the institutional review board of the University Medical Center Utrecht (Utrecht, The Netherlands) under number 10/0225. Informed consent for faecal sampling during hospitalization was waived. Written consent was obtained for the collection of faecal samples after hospitalization.

Data availability

Metagenomic shotgun sequence reads are deposited in the Sequence Read Archive (European Nucleotide Archive) with the primary accession number PRJEB3977. Assemblies can be accessed through MG-RAST with accession numbers 4508944.3, 4508945.3, 4508946.3, 4508947.3 and 4508948.3. Fosmid sequences are deposited at GenBank under accession numbers KF176928–KF176932.

Results

We first monitored the dynamics of the resistome and the gut microbiota composition in a previously healthy patient who had been hospitalized in our hospital’s ICU after a traffic accident. The patient had no history of hospitalization or antibiotic use. The patient received SDD from the first day in ICU for 17 days, after which the patient was transferred to the neurology department, where he remained hospitalized until hospital discharge, 43 days after admittance. Faecal samples were collected at four timepoints during the hospital stay (Days 4, 14 and 16 in ICU and Day 28 in the neurology ward) and at Day 313 (270 days after hospital discharge) (Figure 1a). Diagnostic cultures were performed throughout the patient’s stay in hospital and did not yield any growth of antibiotic-resistant bacteria (Table S1). The antibiotics administered to the patient during hospital stay are shown in Figure 1(a) (further details are provided in Table S2). No antibiotics were prescribed following hospital discharge. Culture-independent techniques were used to profile the diversity of the gut microbiota and its resistome at the five timepoints at which faeces were collected during and after hospitalization.

Expansion of the resistome during ICU stay

The resistome of the patient substantially expanded during the ICU stay and the administration of SDD, this was most pronounced at Days 14 and 16 (Figure 2a). The reservoir of resistance genes had decreased at Day 28, but genes conferring resistance to several classes of antibiotics were still detectable in the absence of antibiotic selective pressure at day 313 (270 days after hospital discharge). Specifically, there was a 6.7-fold

Phylogenetic profiling of patient gut microbiota

16S rRNA gene-based profiling of the gut microbiota revealed that, during hospitalization, the most prevalent groups were Bacteroidetes (ranging from 29% to 67% of the total microbiota) and Clostridium clusters XIVa and IV (from 21% to 69% of the total microbiota), which are all common inhabitants of the intestinal microbiota of healthy humans (Figure 1b; the full dataset is provided as Table S5 and Table S6, both available as Supplementary data at JAC Online). The relative abundance of these three groups fluctuated considerably during hospitalization. Unusually, at Day 28 (11 days after ICU discharge and the discontinuation of SDD), Bacilli represented 10% of the microbiota, which was mainly caused by an increased abundance of enterococci (5.1% of the microbiota). Enterococci are usually quantitatively minor species in the healthy gut microbiota but can become more prominent during hospitalization. At other points in time, Bacilli were less abundant (<1%). The composition of the patient’s microbiota had markedly changed at Day 313 (270 days after hospital discharge). At this timepoint, the gut microbiota consisted almost exclusively of bacteria belonging to the phylum Firmicutes, and was dominated by Clostridium cluster XIVa (87.5% of the total microbiota). Bacteroidetes were present at only 0.67% (Figure 1b). As this patient had not received antibiotics during the 270 days after hospital discharge, this may well reflect the normal, undisturbed state of this particular individual’s microbiota.

Figure 1. Patient history and gut microbiota composition. (a) The timeline indicates the major events throughout the patient’s hospital stay and the times at which faeces were collected. Light green boxes indicate the antibiotics (E, erythromycin; F, flucloxacillin; V, vancomycin; Ce, cefazolin) that were administered to the patient. Further details are provided in the Methods section. Diagnostic culturing was performed for rectum, sputum, throat, urine and blood samples and no antibiotic-resistant bacteria were found at any point in time (Table S1). (b) The patient’s gut microbiota composition was monitored by a 16S rRNA-based microarray profiling approach (HITChip). The bars indicate the relative abundance of the most dominant bacterial phyla in the gut microbiota at the timepoints indicated on the x-axis. The colours code for the different phyla and classes as displayed in the figure key. Low-abundance phyla and classes are grouped together as ‘Others’. Detailed information on the relative abundances of all phyla and classes detected by HITChip analysis are provided in Table S5.
increase in the relative abundance of aminoglycoside resistance genes at Day 16, compared with the first sampling point at Day 4 and the last sampling point at Day 313 (Figure 2). Due to the inclusion of an aminoglycoside (tobramycin) and a β-lactam antibiotic (cefotaxime) in the SDD regimen, we focused on genes that were predicted to confer resistance to these antibiotics. In particular, the aminoglycoside resistance genes *aph(2")-Ib, aph(3')-IIIa* and an *aadE*-like gene increased in abundance during the ICU stay (Figure 2b, Table S4 and Table S7) (available as Supplementary data at JAC Online). In addition, the copy number of the β-lactam resistance gene *cblA* rose during the ICU stay but increased further after ICU discharge (Day 28, Figure 2b, Table S4 and Table S7). Notably, the abundance of aminoglycoside resistance genes was lower at Day 28 and had dropped even further at Day 313 (Figure 2a), although aminoglycoside resistance genes remained the most abundant class of resistance genes in the resistome at this point in time. In addition to aminoglycoside resistance genes, genes conferring resistance to macrolides and tetracyclines were the most abundantly present classes of resistance genes. The abundance of macrolide and tetracycline resistance genes remained relatively stable throughout the hospital stay, but dropped sharply upon hospital discharge. The observed high levels of macrolide resistance genes throughout hospitalization may have been triggered by the usage of erythromycin, which the patient received to enhance gastric emptying during the ICU stay. Tetracyclines were not administered to this patient and the high prevalence of these resistance genes is in line with the previously reported high abundance of tetracycline resistance genes in healthy individuals.27,28

**Resistance genes on mobile genetic elements in anaerobic gut commensals**

Metagenomic shotgun sequencing and subsequent assembly generally resulted in contigs of limited size, precluding an assessment of the genetic context of the identified resistance genes (data not shown). We therefore constructed fosmid libraries (with inserts of ~40 kbp) to functionally screen for antibiotic resistance genes and to further explore the genetic context of these genes.

Five fosmid libraries were constructed in *E. coli* from metagenomic DNA obtained from the faeces samples used for metagenomic sequencing. The total size of these libraries ranged from 0.8 to 2.8 Gbp (Table S8, available as Supplementary data at JAC Online). Libraries were screened for clones that were resistant to ampicillin, cefotaxime, cefazolin, tobramycin, erythromycin, tetracycline and colistin (Figure S1, available as Supplementary data at JAC Online). No clones that were resistant to colistin or cefotaxime were isolated, but we were able to isolate resistant clones for the other antibiotics. The number of clones resistant to tobramycin, ampicillin or erythromycin increased during the ICU stay. At Day 28, the number of tobramycin- and, to a lesser
extent, erythromycin-resistant clones had decreased, whereas the number of ampicillin-resistant clones remained relatively stable, confirming the trends observed by metagenomic shotgun sequencing. The number of tetracycline-resistant clones was relatively stable throughout the monitored period. At Day 313, tetracycline was the only antibiotic for which resistant clones could be isolated (Figure S1). From the resistant clones, five genes were identified that conferred resistance against tobramycin, ampicillin, erythromycin and tetracycline in E. coli. The identified genes were: aph(2’)-Ib (conferring resistance to tobramycin), cblA (ampicillin), ermBP (erythromycin) and tetW and tetO (tetracycline). Sequencing of the vector/insert junction of 10 clones in which resistance genes were identified showed that identical resistance determinants were present within different clones and genetic backgrounds. Subsequently, the inserts of one selected fosmid clone per resistance gene were sequenced to characterize the genetic context of the resistance genes and to predict the bacterial sources of the cloned ISs. This revealed that the cloned resistance genes were harboured by anaerobes from the phyla Firmicutes (Subdoligranulum, Clostridia), Bacteroidetes (Bacteroides uniformis) and Actinobacteria (Figure 3), which are all common members of the human gut microbiota.8,25 In all sequenced fosmid inserts, the antibiotic resistance genes were associated with IS elements or genes of putative plasmid origin, including genes that are predicted to be involved in plasmid replication and mobilization (Figure 3). This suggests that the antibiotic resistance genes are located on mobile genetic elements that are harboured by anaerobic gut commensals.

**Heterogeneous effects of SDD on abundance of aminoglycoside resistance genes in ICU patients**

Because metagenomic sequencing demonstrated an increasing abundance of aminoglycoside resistance genes in the patient’s microbiota during ICU hospitalization, we decided to perform qPCRs to determine the levels of two aminoglycoside resistance genes in 12 additional, ICU-hospitalized patients who were receiving SDD and from whom multiple faecal samples were collected during their ICU stay. The metagenomic DNA samples of the patient of whom the resistome was profiled by metagenomic shotgun sequencing and functional metagenomics was also included. Notably, none of the studied patients was treated therapeutically with an aminoglycoside [Figure 1 and Figure S2 (available as Supplementary data at JAC Online)]. Consequently, the patients’ only exposure to aminoglycoside antibiotics was due to the use of tobramycin in SDD. The two aminoglycoside resistance genes that were targeted by qPCR were aph(2’)-Ib, which was identified in our functional metagenomic screen, and the aadE-like gene, which was the most abundant aminoglycoside resistance gene found by metagenomic shotgun sequencing. The qPCR data indicated that the relative abundance of both resistance genes was highly divergent between the different patients. The copy number of the resistance genes changed between 1.5 × 10⁴ and 8.1 × 10⁻⁸-fold (for aph(2’)-Ib) and 1.0 × 10² and 4.5 × 10⁻¹¹-fold (for the aadE-like gene) relative to the first sampling point during ICU stay (Figure 4). Our findings indicate that the effect of SDD, and ICU hospitalization in general, is highly individualized and that both a strong enrichment and a complete eradication of aminoglycoside resistance genes can be the result of SDD.

**Discussion**

The prophylactic use of antibiotics in SDD is one of the most successful interventions to reduce patient morbidity and mortality in ICU, but whether SDD will lead to the selection of antibiotic-resistant bacteria is a topic of considerable controversy.1,6,7,29 With this study, in which several metagenomic approaches were combined, we provide data indicating that the patient gut microbiota, and the resistance genes carried by the gut microbiota, can be profoundly affected by ICU hospitalization and SDD. Our functional metagenomic analyses indicate that the identified antibiotic resistance genes are all carried by anaerobic gut commensals and are associated with mobile genetic elements.

Based on sequence analysis of a fosmid insert conferring resistance to tobramycin, the aminoglycoside resistance determinant aph(2’)-Ib was harboured by a strain from the genus Subdoligranulum. This genus belongs to Clostridium cluster IV and is commonly present in the microbiota of healthy individuals.8,30 Interestingly, strict anaerobes such as Bacteroidetes and Clostridia are generally thought to be intrinsically resistant to aminoglycosides, because these bacteria lack an electron
transport system that is needed for the energy-driven uptake of aminoglycosides into the cell.\textsuperscript{31} Nevertheless, aminoglycoside resistance genes can be readily identified in several \textit{Clostridium} isolates (including strains that were isolated from human faeces) by either comparative genomic hybridization\textsuperscript{32} or by the sequence analysis of publicly available \textit{Clostridium} genomes (data not shown). These observations not only show that \textit{Clostridium} and closely related genera may serve as a reservoir for aminoglycoside resistance genes, but also suggest that these resistance genes may have a, hitherto unrecognized, function in \textit{Clostridia}. Alternatively, the resistance genes may form part of a larger genetic element that confers a fitness benefit to \textit{Clostridia} and are retained for this reason.

In all sequenced fosmid inserts, we found evidence of the presence of 15 elements and genes of putative plasmid or plasmid origin, including genes that are predicted to be involved in plasmid replication and mobilization. This observation suggests that these resistance genes may be part of larger genetic elements that can be mobilized and/or which have been acquired through horizontal gene transfer. Evidence for the extensive transfer of antibiotic resistance genes in the gut microbiota has been observed before in \textit{Bacteroidetes} and \textit{Firmicutes}.\textsuperscript{10,13} Consequently, the enrichment of antibiotic resistance genes in the patient’s gut microbiota during SDD and their association with mobile genetic elements is a cause of concern as this may facilitate the transfer of resistance genes to aerobic nosocomial pathogens. In fact, our experimental design, using functional metagenomics, proved that these resistance genes can be expressed and are functional in \textit{E. coli}, which is a common cause of hospital-acquired infections.

Based on our findings in a single patient, we subsequently determined the relative abundance of both aminoacyl-sRNA synthetase genes (\textit{aph(2’’)}-Ib and \textit{aadE}-like gene) in 12 other ICU patients who were hospitalized in the ICU for at least 9 days and who received SDD during this period. The relative abundance of both genes appeared very dynamic, indicating highly variable effects of SDD on the studied aminoglycoside resistance genes in individual patients. This may result from differences between the studied patients with respect to the bacterial hosts that carry the antibiotic resistance genes. For instance, the \textit{aph(2’’)}-Ib gene, which was harboured by the Gram-positive bacterium \textit{Subdoligranulum} in the patient in whom we characterized the resistome by metagenomic approaches, can also be harboured by Gram-negative organisms such as \textit{E. coli}.\textsuperscript{34} In addition, the \textit{aadE}-like gene is also found in the genome sequences of both Gram-positive and Gram-negative gut commensals such as \textit{Faecalibacterium prausnitzii} and \textit{Bacteroides uniformis} (data not shown). In patients who carry \textit{aph(2’’)}-Ib in a Gram-negative host, such as \textit{E. coli}, the relatively copy number of this gene may rapidly decrease during SDD due to the action of colistin, as this antibiotic specifically targets Gram-negative bacteria, while not inhibiting the growth of Gram-positive bacteria.

This study suggests that ICU hospitalization and SDD may have a large effect on the gut microbiota and the resistome. Previous, culture-based studies failed to demonstrate that SDD increased the prevalence of colonization by antibiotic-resistant bacteria in the ICU.\textsuperscript{7} This observation indicates that selection for resistance among anaerobic gut commensals during an ICU stay may not directly impact on the resistance levels in aerobic bacteria, possibly because these are eradicated by other components of SDD. However, once patients are discharged from ICU and SDD has been discontinued, the expanded resistome of the patients’ gut microbiota may facilitate the transfer of resistance genes to aerobic pathogens, once these recolonize the patient gut. This mechanism might explain the previously observed increase in antibiotic resistance among Enterobacteriaceae after the cessation of SDD.\textsuperscript{29}

Notably, microbiological cultures that were routinely performed in our diagnostic laboratory failed to yield the growth of any antibiotic-resistant bacterium throughout the period in which this patient was hospitalized. This discrepancy between traditional culture approaches and metagenomic analyses is likely to be due to antibiotic resistance genes being mostly carried by strictly anaerobic gut commensals, which are effectively impossible to culture in routine diagnostic settings. We note that the introduction of metagenomic shotgun sequencing as a tool in clinical diagnostics will allow the comprehensive identification
and quantification of the resistome in individual patients. Although such approaches are currently still restricted by the costs of metagenomic shotgun sequencing and subsequent data analysis, our findings highlight the potential of these approaches as a future monitoring tool for assessing the impact of antibiotics on the gut resistome and to guide personalized antibiotic treatment. Most importantly, our findings indicate that the benefits of SDD on patient outcome and infection rates must be carefully balanced against the potential collateral selection and amplification of antibiotic resistance genes among anaerobic gut commensals.

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Supplementary data
Tables S1 to S8, Figure S1 and Figure S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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