Interspecies effects in a ceftazidime-treated mixed culture of *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Staphylococcus aureus*: analysis at the single-species level

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Objectives: *In vitro* studies commonly use single bacterial isolates for testing antibiotic susceptibilities. However, interspecies effects that may arise when mixed infections are treated with antibiotics can obviously not be investigated by this approach. In the study presented here, the effect of ceftazidime against a model microbial community consisting of *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Staphylococcus aureus* was studied in order to reveal effects that only may appear in a ceftazidime-treated mixed culture.

Methods: Time–kill experiments were conducted with mixed and pure cultures in a defined medium containing 30 mg/L ceftazidime. Interspecies effects were revealed by comparing growth and kill dynamics from time–kill experiments with results from untreated mixed and pure cultures. For species-specific cell enumeration, a quantitative terminal restriction fragment length polymorphism was used. Ceftazidime was measured by HPLC.

Results: *P. aeruginosa* showed only a lytic phase in the ceftazidime-treated mixed culture, but not in the untreated mixed culture nor in the ceftazidime-treated pure culture. On the other hand, *S. aureus* did not lyse in the ceftazidime-treated mixed culture, while it did in the untreated mixed culture.

Conclusions: This finding suggests that the efficacy of ceftazidime against *P. aeruginosa* was increased by an interspecies effect during co-cultivation with *B. cepacia* and *S. aureus*. The latter seemed to be negatively affected by interspecies effects in mixed culture without ceftazidime. The same effect was nullified when ceftazidime was applied to the mixed culture. Further studies are required to reveal the underlying mechanisms.

Keywords: T-RFLP, time–kill curves, cystic fibrosis, cell lysis

Introduction

*In vitro* antibiotic susceptibility testing is routinely conducted for the determination of MIC, which describes the efficacy of an antibiotic to inhibit the visible growth of bacterial isolates under standard conditions. However, this approach is limited in its prediction power for many infections that may occur in humans or animals. For example, infections can occur as consortia of two or more bacterial species1–3 and interactions between the microorganisms might affect the efficacy of antibiotic treatment, with consequences for therapeutic treatment. Obviously, both scenarios are not covered by routine susceptibility testing with single bacterial isolates.

Interactions between different microorganisms, which can impact on antibiotic susceptibility, may occur in consortia involving bacteria expressing β-lactamases and bacteria that are not able to express β-lactamases.4–7 This co-pathogenicity may arise by a protecting effect, in which the non-β-lactamase producers have a higher chance of surviving treatment with β-lactam antibiotics8 due to the reduced antibiotic activity afforded by the β-lactamase producers. This was confirmed by studies in time–kill experiments for mixed cultures consisting of aerobic and anaerobic bacteria9,10 and for a mixed culture comparable to the normal flora of the nasopharynx.11 Further studies investigated strains from the same species, but with different resistance properties in terms of bacterial competition under antibiotic stress. In particular, the impacts of antibiotic resistance as a protecting mechanism and as a burden to bacterial fitness were characterized.12–14 In the corresponding studies, the population distribution changed significantly as a consequence of antibiotic treatment. Thus, strain-specific resistance mechanisms seem to be a prerequisite for gaining dominance in a mixed culture under antibiotic stress. Whether
antibiotic resistance can also constitute a burden for growth in an antibiotic-free environment could not be shown clearly.

Only a few studies have assessed antibiotic activity against mixed cultures at the single-species level. Most of them investigated the change in viability in so-called time–kill curves. These offer an advantage over routine susceptibility tests, as they evaluate both the rate and the extent of bacterial killing over time. In the case of mixed cultures, time–kill curves allow the obtention of additional information regarding changes in population distributions with time and the identification of trends in the composition of the bacterial consortia. In all the studies mentioned above, a selective spread-plate method was used to distinguish single species in a culture and to determine their concentration. This method comprises serial dilution, followed by plating on agar plates with selective media or agents, which allow the replication of only one or more bacterial species with suitable growth characteristics. Discrimination between different bacterial species can also be achieved by aerobic and anaerobic incubation of the spread plates. However, the methodology has some clear disadvantages: (i) there is a high work load involved with the double serial dilution of each sample for statistical significance; (ii) the number of plates is further increased by the number of bacterial strains contained in the sample; (iii) the species in the sample must be known and very well characterized in terms of growth characteristics for the choice of specific growth conditions; and (iv) the colony-forming ability is not only influenced by cell concentration and viability, but also by morphological alterations after exposure to antibiotics.

A possibility to avoid these disadvantages is offered by the use of the quantitative terminal restriction fragment length polymorphism (qT-RFLP) method, 21,22 which allows species-specific cell enumeration in samples with two or more bacterial species. The method is based on molecular biological techniques for the quantification of genome copies extracted from a sample. Discrimination for a single species is based on their specific 16S rRNA gene, which leads to different DNA fragment lengths after a restriction digestion. Similar techniques are commonly used in environmental biology for the characterization of bacterial diversity in complex consortia. 23 The method is easily transferable and can be used, besides other techniques like flow cytometry, as a platform technology for various mixed culture applications without extensive method optimization. Furthermore, the work load for sample preparation (see the ‘Materials and methods’ section) does not increase with the number of microorganisms in a sample. Thus, qT-RFLP is the method of choice when more than two or an unknown number of microorganisms are to be quantified.

In this study, we examined the dynamics of the ceftazidime-induced killing of a microbial mixed culture consisting of the Gram-negative bacteria Pseudomonas aeruginosa and Burkholderia cepacia, and the Gram-positive bacterium Staphylococcus aureus. As a control, all three microorganisms were cultivated additionally in mixed culture without antibiotic, as well as in pure culture with and without antibiotic. The bacteria were chosen due to their medical relevance as a model for a community that occurs in the lungs of many patients with cystic fibrosis. Ceftazidime, the antibiotic used here, is a cephalosporin with a high efficacy against P. aeruginosa. It is also known to induce filament formation in Gram-negative bacteria, which negatively influences the results of the common spread-plate method. 24 To our knowledge, it is the first report of the qT-RFLP method being used for investigations of growth dynamics of bacterial mixed cultures under the influence of an antibiotic.

Materials and methods

qT-RFLP fragment analysis method

qT-RFLP for absolute and species-specific cell enumeration was performed as previously described by Schmidt et al. In the following, only a brief overview is given.

Aliquots (100 µL) of Campylobacter jejuni with a cell concentration of 2.0 × 10^9 cfu/mL were used as the internal quantification standard (IQS). At each timepoint, two samples were taken and analysed in parallel (technical duplicate). A sample was prepared by adding 1 mL of culture to an IQS aliquot. After centrifugation and washing with PBS, the DNA of the bacterial mixture was extracted by enzymatic cell lysis followed by DNA purification with a commercially available kit (QIAamp DNA Blood Mini Kit, Qiagen, Germany). The 16S rRNA genes of the extracted and purified DNA were amplified using a gene-specific and fluorescently labelled primer. After purification in an agarose gel, the amplicon was restricted with an HhaI endonuclease. A Genetic Analyzer ABI Prism 3100Avant (Applied Biosystems) was used for separation of species-specific DNA fragment lengths, which were quantified by laser-induced fluorescence. The ratio of the detected peak area for the species of interest and the peak area of the IQS was used to determine the absolute cell number (N) for this species 21,22 in units of genome equivalents per mL (ge/mL). This unit is equivalent to cfu/mL under antibiotic-free cultivation conditions. On the other hand, when ceftazidime is applied, the formation of colonies is influenced strongly by morphological alterations and the expression of genome equivalents in cfu/mL could result in the misinterpretation of experimental data (also see the ‘qT-RFLP as quantification method assessing growth and lysis in time–kill experiments’ section). Therefore, cell concentrations were expressed in ge/mL throughout this study.

Furthermore, the absolute cell concentration N_t at timepoint t was normalized by the absolute cell concentration at timepoint zero (N_0) and expressed as the logarithm to the base 2 of (Equation 1):

\[ \text{Number of cell doublings} = \log_2 \left( \frac{N_t}{N_0} \right) \]  

The result is equivalent to the number of cell doublings at a given timepoint and was plotted against time as a measure of the relative growth of single microorganisms. This facilitates a comparison of the growth dynamics of single microorganisms in mixed and pure culture experiments, despite differences in absolute cell concentrations, which were a consequence of the inoculation method (see ‘Inoculum preparation and time–kill experiments’ section).

Culture medium

M199 cell culture basal medium powder (without NaCO₃) (Gibco, Karlsruhe, Germany) was used as a chemically defined, but rich (full) medium without undefined (complex) components. Different from the manufacturer’s recommendation, phosphate instead of carbonate was used as a buffer. Additionally, nitrilotriacetic acid (NTA) was added to prevent precipitation and allow efficient growth of bacterial strains under investigation. So, 800 mL of ultrapure water, 16 mL of NTA solution [0.25 mM NTA (AppliChem, Darmstadt, Germany) in 0.6 M NaOH (Merck, Darmstadt, Germany) solution] and 25 mL of sodium potassium phosphate buffer (1.5 M NaH₂PO₄/K₂HPO₄, pH 7.0; Roth, Karlsruhe, Germany) were mixed with the amount of powder indicated by the supplier and completed to final volume with ultrapure water.
Bacterial strains and antibiotic agent

In this study, *P. aeruginosa* PA01, *B. cepacia* DSM 7288 and *S. aureus* ATCC 29213 were used.

Ceftazidime (Molekula, Shaftesbury, UK) was used as the antibiotic agent. A stock solution with 2 g/L ceftazidime in culture medium was freshly prepared and sterile filtered prior to experiments. The MIC was determined for each microorganism using a microdilution assay according to DIN 58940-81, with supplemented culture medium M199 and *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *P. aeruginosa* ATCC 27853 as control strains. The following MIC values were determined: *P. aeruginosa* PA01, 2.0 mg/L; *B. cepacia* DSM 7288, 32 mg/L; and *S. aureus* ATCC 29213, 8.0 mg/L. The value for *P. aeruginosa* PA01 and *B. cepacia* DSM 7288 (= *B. cepacia* LMG 1222) is consistent with other studies.27–29

The first-order degradation constant of ceftazidime was determined to 

\[ k_{\text{ceftazidime}} = -0.0072 \text{ h}^{-1} \] under the same experimental conditions (modified medium M199, 37°C, without microorganisms).

Inoculum preparation and time–kill experiments

All cultures were grown in 250 mL wide-neck Erlenmeyer flasks and incubated in a humidified orbital shake incubator (Kuehner, Birsfelden, Switzerland) at 37°C, rotation speed 200 rpm, eccentric radius 1.25 cm and relative humidity 85%.

For inoculum preparation, all three strains were cultivated separately overnight in shake flasks with modified medium M199 (20 mL). The harvested cells were centrifuged (4800 g, 4°C, 10 min) and washed with PBS (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L NaH₂PO₄, 0.2 g/L K₂HPO₄). After a second centrifugation step, strains were suspended in 20 mL of fresh medium and cultivated for an additional 1.5 h. For pure culture time–kill experiments, 630 µL of these exponentially growing cultures were inoculated in 50 mL pre-warmed medium (30 mg/L ceftazidime). For mixed culture time–kill experiments, 210 µL of each strain (ratio 1:1:1) was inoculated into the same volume of ceftazidime-containing medium. All cultivations were performed in triplicate to increase statistical significance. The total starting cell concentrations in all pure and mixed culture time–kill experiments were in the range of \( 10^{8} \text{–} 10^{9} \text{ cfu/mL} \) which is equivalent to \( 10^{9} \text{–} 10^{10} \text{ cfu/mL} \) under antibiotic-free cultivation conditions.

Analysis of antimicrobial agent

Ceftazidime concentration was determined via HPLC (Agilent 1200 Series (Santa Clara, CA, USA); detector, diode array DAD G1315B (Agilent, USA); UV wavelength, 256 nm; column, Hypersil Gold 150 × 4.6 mm (Thermo Fisher Scientific, Dreieich, Germany); eluents, 5 g/L (NH₄)₂HPO₄ [pH = 3.90 adjusted with H₂PO₄ (Merck)] and acetonitrile (ACN) (Roth); gradient profile, 10 min isocratic 1.8% ACN + 98.2% (NH₄)₂HPO₄, linear gradient to 10% ACN + 90% (NH₄)₂HPO₄ until 25 min total run time, isocratic 10% ACN + 90% (NH₄)₂HPO₄ until 30 min total run time and isocratic 1.8% ACN + 98.2% (NH₄)₂HPO₄ until 35 min total run time; flow, 1.0 mL/min; temperature, 40°C; and injection volume, 10 µL). Ceftazidime standards were freshly prepared for each experiment and stored under the same conditions as the samples. Each sample was filtered through 0.2 µm syringe filters (Spartan 13/RC; Whatman GmbH, Dassel, Germany) immediately after sampling and stored at 4°C until measurement.

Results

Two mixed culture time–kill experiments were performed on different days: one without the addition of ceftazidime; and the other with the addition of 30 mg/L ceftazidime at the start of cultivation. For each bacterial strain, a triplicate of pure cultures was cultivated in parallel under identical experimental conditions.

Pure and mixed culture growth behaviour without ceftazidime

During pure culture growth, *P. aeruginosa* doubled its cell concentration ~6 times during the first 7 h, without any lag-phase (Figure 1a). This was clearly higher than the growth of *B. cepacia* and *S. aureus* under the same cultivation conditions. Both doubled their cell number only ~3.5 times during the first 9 h of growth. In mixed culture growth, *P. aeruginosa* showed enhanced growth behaviour regarding cell doublings (~7.6) over a slightly extended total growth phase (exponential growth phase + transition phase) of 9 h (Figure 1b). In contrast, *S. aureus* showed a decreased maximum doubling number of about two compared with the single cultivation. Towards the end of the experiment, the cell concentration of *S. aureus* even decreased to almost the value at the time of inoculation. The third bacterium (*B. cepacia*) grew under both conditions equally well, without any significant alterations in growth dynamics.

Time–kill curve of the single species

To investigate the effect of ceftazidime on the growth characteristics in pure culture, each bacterium was cultivated with 30 mg/L ceftazidime in three parallel batches (Figure 1c). This concentration corresponded to 15 times the MIC value of *P. aeruginosa*, ~4 times the MIC value of *S. aureus* and 1 times the MIC value of *B. cepacia*. Cultivations of single bacteria without ceftazidime served as a control (Figure 1a).

The shape of the time–kill curve of the antibiotic-treated *P. aeruginosa* pure culture was similar to the growth curve in untreated culture (Figure 1a). The maximum specific growth rates were approximately the same (Table 1, \( t \)-test for comparison of linear regression coefficients; \( P \)-value = 0.142); although, the total growth phase was reduced to ~3 h compared with 5 h in the untreated, pure culture. The maximum doubling number of 5 remained constant until the end of the experiment (24 h), yet it was reduced by 1 compared with the untreated pure culture (maximum 6 doublings). A clear kill phase (in terms of cell lysis) was not evident.

In antibiotic-treated *B. cepacia* pure culture (Figure 1c), the maximum specific growth rate (Table 1, \( P \)-value = 0.332), as well as the length of the total growth phase (~8 h), did not differ significantly from the untreated pure culture (Figure 1a). However, there was a slight but significant decrease in the doubling number (consistent with a drop in absolute cell concentration) afterwards (7 h until ~24 h) (Figure 1c), which may be interpreted as cell lysis due to ceftazidime treatment.

In ceftazidime-treated *S. aureus* pure culture (Figure 1c), a comparably short total growth phase of 2 h led to a reduced maximum doubling number of ~2 (reduction by 1.5 doublings compared with untreated pure culture (Figure 1a)) and remained constant until the end of cultivation without any indication of cell lysis.
In order to reveal interspecies effects under the antibiotic regimen of 30 mg/L ceftazidime, time–kill curves of all three species were determined by co-cultivation (Figure 1c).

Furthermore, results were compared with mixed culture results without ceftazidime administration (Figure 1b), which served as control for interspecies effects without the influence of ceftazidime.

Table 1. Maximum specific growth rates $\mu_{\text{max}}^\circ \pm 95\%$ confidence interval in the time interval $\Delta t$

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pure culture $\mu_{\text{max}}$ 1/h</th>
<th>$\Delta t$ (h)</th>
<th>Mixed culture $\mu_{\text{max}}$ 1/h</th>
<th>$\Delta t$ (h)</th>
<th>Pure culture +30 mg/L ceftazidime $\mu_{\text{max}}$ 1/h</th>
<th>$\Delta t$ (h)</th>
<th>Mixed culture +30 mg/L ceftazidime $\mu_{\text{max}}$ 1/h</th>
<th>$\Delta t$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>0.34 ± 0.09</td>
<td>0–4.1</td>
<td>0.40 ± 0.07</td>
<td>0–3.0</td>
<td>0.53 ± 0.12</td>
<td>0–3.0</td>
<td>0.67 ± 0.16</td>
<td>0–3.0</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>0.14 ± 0.05</td>
<td>0–9.0</td>
<td>0.28 ± 0.08</td>
<td>0–3.0</td>
<td>0.11 ± 0.04</td>
<td>0–7.0</td>
<td>0.30 ± 0.10</td>
<td>0–3.0</td>
</tr>
<tr>
<td>S. aureus</td>
<td>0.14 ± 0.03</td>
<td>0–9.0</td>
<td>0.25 ± 0.10</td>
<td>0–3.0</td>
<td>0.30 ± 0.13</td>
<td>0–2.0</td>
<td>0.28 ± 0.18</td>
<td>0–3.0</td>
</tr>
</tbody>
</table>

*aBy linear regression, Matlab® (Version 7.9), curve fitting toolbox 2.1.

**Time–kill curve of three-species mixed culture**

In order to reveal interspecies effects under the antibiotic regimen of 30 mg/L ceftazidime, time–kill curves of all three species were determined by co-cultivation (Figure 1d). The time–kill curves of single bacteria from pure culture, which were cultivated in parallel (Figure 1c), served as control. Furthermore, results were compared with mixed culture results without ceftazidime administration (Figure 1b), which served as control for interspecies effects without the influence of ceftazidime.
The time–kill curve of \textit{P. aeruginosa} in antibiotic-treated mixed culture (Figure 1d) started with the same trend as that observed within all of the other experiments presented here. The total growth phase lasted \( \sim 3 \) h and led to a maximum doubling number of \( \sim 6 \). The maximum specific growth rate was not significantly different from the growth rate of the untreated mixed culture (Table 1, \( P \text{ value}=0.096 \)) or the ceftazidime-treated pure culture (Table 1, \( P \text{ value}=0.283 \)) during the first 3 h. Taking into account the uncertainty of the measurements, the maximum specific growth rates in all three experimental set-ups were equal. Also, the duration of the total growth phase was similar to the ceftazidime-treated pure culture (Figure 1c). In the untreated mixed culture (Figure 1b), an extended total growth phase was observed until \( \sim 9 \) h, resulting in a higher cell concentration that is consistent with \( \sim 7.6 \) cell doublings. A clear difference from the ceftazidime-treated pure culture was found in the time interval between 3 and 24 h. There was a decrease in the doubling number, which reached a final value of one doubling, while in the ceftazidime-treated pure culture the doubling number remained constant during the whole stationary phase.

\textit{B. cepacia} doubled \( \sim 4 \) times during the first 5 h in antibiotic-treated mixed culture (Figure 1d) with a significantly higher (\( P \text{ value}=0.022 \)) maximum specific growth rate (Table 1) than in the antibiotic-treated pure culture (Table 1). However, the maximum cell concentration reached in both cultures was similar (\( P \text{ value}=0.162 \), Table 2). The number of cell doublings began to decrease at \( \sim 7 \) h in both cultures. The last data point (at 24 h) indicated a restart of cell growth only in the antibiotic-treated mixed culture. Compared with the untreated mixed culture (Figure 1b), no significant differences in the dynamics of cell doubling number were identified. However, this was predominantly influenced by the relatively high standard deviation of the values in the untreated mixed culture.

\textit{S. aureus} reached a maximum doubling number of \( \sim 3.5 \) in the ceftazidime-treated mixed culture (Figure 1d) during the first 4 h of cell growth. This was a significantly higher maximum doubling number (\( P \text{ value}=0.014 \)) than in pure culture (Figure 1c), which showed about two doublings during the first 2 h of cell growth. Even though \textit{S. aureus} doubled more often in the treated mixed culture, a lower maximum absolute cell concentration was obtained (\( 10^6.85 \), Table 2). In this context, the lower inoculation concentration in the treated mixed culture (Figure 1c: \( N_{\text{Sa}, \ t=0}=10^6.75 \) ge/mL; Figure 1d: \( N_{\text{Sa}, \ t=0}=10^5.78 \) ge/mL) may have played a role, i.e. a higher number of doublings was achieved until further growth was limited by depletion of substrates, antibiotic killing or other effects.

**Table 2. Maximum cell concentrations± SD**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pure culture (ge/mL)</th>
<th>Mixed culture (ge/mL)</th>
<th>Pure culture + 30 mg/L ceftazidime (ge/mL)</th>
<th>Mixed culture + 30 mg/L ceftazidime (ge/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{P. aeruginosa}</td>
<td>( 10^6.54±0.58 )</td>
<td>( 10^6.24±0.29 )</td>
<td>( 10^7.11±0.36 )</td>
<td>( 10^8.81±0.51 )</td>
</tr>
<tr>
<td>\textit{B. cepacia}</td>
<td>( 10^7.68±0.35 )</td>
<td>( 10^6.81±0.49 )</td>
<td>( 10^7.12±0.19 )</td>
<td>( 10^7.02±0.25 )</td>
</tr>
<tr>
<td>\textit{S. aureus}</td>
<td>( 10^6.15±0.29 )</td>
<td>( 10^6.81±0.27 )</td>
<td>( 10^7.36±0.10 )</td>
<td>( 10^6.85±0.28 )</td>
</tr>
</tbody>
</table>

**Figure 2. Ceftazidime concentrations in time–kill experiments with pure and mixed cultures (also see Figure 1c and d). The theoretical ceftazidime degradation was calculated with the degradation constant \( k_{\text{ceftazidime}}=-0.0072 \) 1/h (determined in an independent experiment, data not shown). Error bars show the mean±SD of three parallel cultivations.**

**Ceftazidime concentration during time–kill experiments**

The ceftazidime concentration was measured during all time–kill experiments (Figure 2). The decrease in ceftazidime concentration in pure cultures of \textit{P. aeruginosa} and \textit{S. aureus} corresponded to the decrease expected for chemical degradation in microorganism-free medium (Figure 2). However, the ceftazidime concentration of the antibiotic-treated pure culture of \textit{B. cepacia} dropped much faster compared with all other experiments and could not be explained by chemical degradation alone. This clearly suggests that \textit{B. cepacia} expressed one or more \( \beta \)-lactamases. Furthermore, the ceftazidime concentration in mixed culture did not decrease as fast as in the pure culture of \textit{B. cepacia}. Again, this decrease is most likely to be related to the release of \( \beta \)-lactamases by \textit{B. cepacia} fraction of this microbial community.

**Discussion**

**Mixed culture growth**

Assuming similar growth dynamics, complete substrate consumption and substrate competition as the only interaction, the biomass yield of single microorganisms will be lower in mixed culture compared with pure culture. In this study, this correlation was observed for the growth of \textit{P. aeruginosa}: the maximum absolute cell concentration was significantly
decreased (P value=0.035) in mixed culture (10^{8.24} \text{ ge/mL}, Table 2) compared with pure culture (10^{8.54} \text{ ge/mL}, Table 2), yet the maximum doubling number (Figure 1a and b) was increased in mixed cultivation. At first glance, this result seems to be contradictory. However, it can be explained by the larger cell concentration at the time of inoculation of the single microorganism in the mixed culture, which allowed for a higher number of cell divisions before substrates were depleted. S. aureus, on the other hand, had a reduced maximum doubling number in mixed culture (Figure 1a) compared with pure culture (Figure 1b). This limitation effect might be a consequence of substrate competition that occurred in co-culture with the other microorganisms. This interpretation is supported by the fact that the glutamine and glucose concentrations dropped below the detection limit after the first 3 and 6 h, respectively (data not shown). Another reason for the reduction in the number of cell doublings may, of course, be related to unknown interactions of P. aeruginosa and/or B. cepacia with S. aureus in the mixed culture experiment.

While substrate competition seems to be the simplest and most likely explanation for the reduced maximum cell concentrations of S. aureus in mixed culture growth (Table 2), it does not explain the reduction in the cell concentration towards the end of cultivation (9 h until 24 h, Figure 1b). This cell lysis at the end of the mixed culture (Figure 1b) was in clear contrast to the constant cell concentration of S. aureus in pure culture during the same time period (Figure 1a). This is a clear indication for an interspecies effect that negatively influenced the growth of S. aureus, with cell lysis as a consequence. This result fulfils either the criteria for amensalism or eccrinolysis. The first denotes an interaction in which one microorganism negatively influences the growth of another microorganism by producing toxins or inhibitors. The second, ecrinolysis, describes an interaction of one microorganism that is being negatively affected by lytic agents produced by the second microorganism, which in turn benefits from additional growth substrates released from the lysed counterpart. Here, no positive effects like additional growth of P. aeruginosa and/or B. cepacia could be identified. A moderate increase in cell concentrations, however, cannot be excluded completely due to the relatively high standard deviation of the measurements. Nevertheless, the observed interaction is presumably of the amensalism type. In this case, cell lytic activity could be related to the formation of membrane vesicles (MVs), which contain cell wall-degrading enzymes and are constitutively released by Gram-negative bacteria. In particular, it is known that P. aeruginosa is able to kill S. aureus after the attachment of its MVs onto the surface of S. aureus and the release of the contained enzymes. Among the components released are a 26 kDa peptidoglycan hydrolase (autolysin) and a LasA protease. Both enzymes exhibit lytic activity against the peptidoglycan layer of S. aureus. On the other hand, B. cepacia might have also played a role in the observed lytic activity against S. aureus, as strains from the genomovars IIIa and V are able to release MVs containing several peptidoglycan-degrading enzymes as well. So far, it has not been demonstrated that this is also true for the B. cepacia strain used in this study (genomovar I), but it seems not to be unlikely.

**qT-RFLP as quantification method assessing growth and lysis in time–kill experiments**

The administration of 30 mg/L ceftazidime in the pure cultures (Figure 1c) showed a common trend for all three microorganisms; the maximum cell concentration (determined as ge/mL by qT-RFLP) was reduced and there was no distinct kill phase or regrowth phase, except for B. cepacia. At first, this may seem to be contradictory to the findings of other authors, who clearly observed kill and regrowth phases in time–kill studies with P. aeruginosa and S. aureus strains under ceftazidime treatment. However, in these studies, a viable cell count method was used, which interprets the number of colonies (cfu/mL) after incubation of a diluted sample on an agar plate as the number of viable cells in the sample. Besides the viability, spread plate-derived results are also influenced by the morphological alterations triggered by β-lactam antibiotics, like ceftazidime. The antibiotic activity may cause spheroplast (oval or round-shaped cells) or filament formation. In both cases, the actual cell concentration is not correctly represented by colony formation on agar plates. Filaments containing numerous bacteria may only form a single colony. In a time–kill experiment, the filaments can even become larger over time due to cell growth, with the consequence that the discrepancy between cfu and actual cell concentration increases. In contrast, the qT-RFLP method used here assesses the cell concentration by the number of genomes that can be extracted from the sample, without being influenced by changes in morphology. The use of viability or genome equivalent-based assays may therefore result in different findings, even when the same system is being investigated.

**Interspecies effect in ceftazidime-treated mixed culture**

Under identical cultivation conditions, cell lysis of antibiotic-treated P. aeruginosa was found only in mixed culture (Figure 1d) and not in pure culture (Figure 1c). This suggests

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**Table 3. Qualitative cell lytic behaviour in all experiments**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pure culture</th>
<th>Mixed culture</th>
<th>Pure culture + 30 mg/L ceftazidime</th>
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</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>S. aureus</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

–, no cell lysis; +, –, no clear result; +, distinct cell lytic phase; ++, distinct cell lytic phase with high activity.
that lysis in mixed culture is a consequence of an unknown inter-
species effect. Additionally, there was no lysis of P. aeruginosa
observable in the mixed culture without ceftazidime
(Figure 1b). Therefore, not only is co-cultivation with B. cepacia
and S. aureus a prerequisite for the lytic effect, but also the pres-
ence of ceftazidime seems to be necessary. Apparently, there
was a higher effective lytic activity against P. aeruginosa in the
ceftazidime-treated mixed culture (Table 3). On the one hand,
this might have been induced by an increased production or
release of lytic enzymes by B. cepacia or S. aureus. On the other
hand, a decreased resistance against lytic enzymes,
which are intrinsically produced by B. cepacia (genomovars IIIa
and V) and released in the form of MVs, could also have had
an impact. In any case, lytic activity seemed to play a key role.
This leads to the question why S. aureus and B. cepacia were
not affected in the same way as P. aeruginosa. Moreover,
results even indicated improved growth properties in terms of
maximum doubling numbers compared with pure cultures for
both microorganisms treated with ceftazidime.

S. aureus did not lyse in ceftazidime-treated co-cultivation,
although this happened in the co-cultivation without ceftazidime
(Figure 1b and Table 3). Thus, it seems that lytic activity against
S. aureus might be reduced in ceftazidime-treated mixed culture.
This would fit into the context of P. aeruginosa being an antag-
onist against S. aureus, with an amensalism type of interaction (see
above). In particular, P. aeruginosa itself was strongly affected by
lysis under these experimental conditions and therefore was not
able to produce lytic activity against S. aureus.

Finally, for B. cepacia, no clear trend was observed in the
ceftazidime-treated mixed culture. A decrease of cell concen-
tration in the time interval 7–12 h was followed by an increase
at 24 h. However, both trends are described by four data points
only and additional experiments would be required to further
investigate this phenomenon.

The degradation rate of ceftazidime is influenced by tempera-
ture and chemical background. In addition, enzymes for the
degradation of β-lactam antibiotics (lactamases) can be expres-
sed by many bacteria, which can result in the protection
of other bacteria against these antibiotics. Most likely, the
decrease in ceftazidime concentration during time–kill exper-
iments with B. cepacia was due to lactamase expression
(Figure 2: B. cepacia pure culture and mixed culture). However,
it is questionable whether this provided a protective function
by reducing the antibacterial pressure for the other bacteria
during mixed culture. In particular, the ceftazidime concen-
tration was always higher than the MIC concentration of
P. aeruginosa (MIC: 2.0 mg/L) and S. aureus (MIC: 8.0 mg/L).
Thus, it seems unlikely that the decline of ceftazidime concen-
tration had a measurable impact on the dynamics of killing or
lysis of the two strains.

Conclusions
In the presented study, a novel approach for investigating the
impact of ceftazidime administration in mixed cultures was
used. Results obtained with qT–RFLP, a molecular biologically
based method for species-specific quantification of microbial
communities, revealed various interspecies effects in co-
cultivation experiments with and without ceftazidime. Com-
parison of the growth dynamics indicated that cell lysis played
a key role, depending on co-cultivation and ceftazidime
exposure. In the presence of ceftazidime, P. aeruginosa lysed
only during co-cultivation with B. cepacia and S. aureus, but
neither in pure culture with ceftazidime nor in mixed culture
without ceftazidime. Apparently, both conditions were necessary
for the observed phenomenon. This also suggests that unknown
interspecies mechanisms can increase the efficacy of ceftazi-
dime against P. aeruginosa. On the other hand, S. aureus was
not affected by lysis in the ceftazidime-treated mixed culture,
while it was in the untreated mixed culture. Whether there is a
causal correlation between the lytic phenomenon of P. aerugi-
nosa and S. aureus must be answered in additional studies.

A protecting effect that was potentially possible due to the
production of β-lactamases with ceftazidime-degrading activity
by B. cepacia did not seem to play a role.

The use of the qT–RFLP method resulted in new insights con-
cerning growth and lysis in a mixed culture model treated with
β-lactam antibiotics. A major advantage is that results were
independent of morphological alterations, which often occur
after the administration of β-lactam antibiotics.
Ceftazidime-treated mixed cultures


