INTRODUCTION

The oligomorphic yeast *C. albicans* can cause localized superficial infections as well as life-threatening systemic candidiasis in immunocompromised individuals. *C. albicans* infections accompany severe diseases, are hospital-acquired, in the majority of cases, and are the fourth most frequent cause of nosocomial sepsis. Thus they have become a major complication particularly in intensive care units (Pfaller & Diekema, 2007).

Fludioxonil is a phenylpyrrol fungicide that is widely used in agriculture, especially in the protection of grapes and berries from plant-pathogenic fungi, most prominently *Botrytis cinerea*. While fludioxonil is highly toxic to some aquatic organisms, the toxicity to mammals has been low or negligible in a wide range of toxicological studies. The fungicide interacts with the osmotic stress response of filamentous fungi and yeasts by inhibiting the activity of a type III histidine kinase (Pillonel & Meyer, 1997; Okada et al., 2005; Ochiai et al., 2002). A homologous histidine kinase, termed Nik1p, is expressed in the opportunistic human-pathogenic yeast *Candida albicans* (Alex et al., 1998; Yamada-Okabe et al., 1999) and is also targeted by fludioxonil (Buschart et al., 2012). Furthermore, Nik1p has been implicated in the yeast–hyphae transition (Alex et al., 1998; Yamada-Okabe et al., 1999), which is a major pathogenicity factor (Calderone & Fonzi, 2001). Therefore, we investigated the effect of fludioxonil on *C. albicans*. In a comprehensive gene expression analysis, we observed that fludioxonil induced the expression of the ATP-binding cassette (ABC) transporters Cdr1p and Cdr2p.

The overexpression of such transporters can cause a higher resistance to antymycotic drugs, as the intracellular concentration of the antymycotic is decreased (Wirsching et al., 2000; Siikala et al., 2010). Active transport by ABC transporters, especially of the PDR-subfamily, is achieved by coupling ATP-hydrolysis to export (Prasad et al., 2000; Sipos & Kuchler, 2006). The genome of *C. albicans* contains nine genes for PDR-subfamily ABC transporters, according to Gaur et al. (2005) and the *Candida* Genome Database (Arnaud et al., 2012), and the relevance of the transporters Cdr1p and Cdr2p in resistance to fluconazole has been studied intensively (Prasad et al., 1995; Sanglard et al., 1997).

Fluconazole is one of the azole antymycotics, which are used to treat systemic *C. albicans* infections, besides polyenes, echinocandins and flucytosin. Acquisition of
resistance to one or several of these antimycotics has been observed (Rex et al., 1995; Fournier et al., 2011). Resistance to fluconazole is usually found in <10% of clinical isolates of C. albicans but the proportion of fully susceptible strains is <80% (Sanglard & Odds, 2002; Pfaffer & Diekema, 2007).

Cdr1p seems to be more important in fluconazole resistance than Cdr2p (Tsao et al., 2009). Besides fluconazole, Cdr1p is able to export human hormones, like oestradiol (Krishnamurthy et al., 1998a), and phospholipids (Dogra et al., 1999; Smriti et al., 2002; Shukla et al., 2007). A higher susceptibility of a cdr1 mutant pointed to Cdr1p’s activity in the efflux of terbinafine, cycloheximide, brefeldin A and fluphenazine (Sanglard et al., 1996).

Constitutive overexpression of CDR1 and CDR2 genes is caused by mutations of the transcriptional regulator Tac1p (Coste et al., 2004, 2006). Transient upregulation of CDR1 and CDR2 genes, which also attenuate susceptibility to antimycotics, has been observed upon biofilm formation and heat shock, or exposure to hormones and some antimycotic compounds as well as agricultural herbicides (Krishnamurthy et al., 1998b; Liu et al., 2005; Schmidt et al., 2008).

Hence, we studied the effect of fludioxonil on the expression of CDR1 and CDR2 genes in C. albicans. We found that the induced expression of the CDR1 and CDR2 genes during exposure to fludioxonil was a target-independent side effect. Therefore, we also investigated the consequences of fludioxonil exposure for the export efficiency of antimycotic compounds and the susceptibility of C. albicans to these compounds.

METHODS

Organisms and culture conditions. C. albicans isolates ATCC-10231, DSM-1577 (both obtained from the DSMZ – German Collection of Microorganisms and Cell Cultures) and SC5314 (ATCC MYA-2876; Gillum et al., 1984), as well as the SC5314-derived deletion mutants Δcdr1 (Nobile & Mitchell, 2009), Δcdr2 (Sanglard et al., 1997), Δcdr1Δ2 (Sanglard et al., 1997), Δtac1 (Homan et al., 2009) and Δniki (Alex et al., 1998), were used in this study. Overnight cultures were prepared in 250 ml flasks with 50 ml YPD medium at 30 °C. Pre-cultures were prepared by diluting the overnight cultures in 20 ml YNB (yeast nitrogen base without amino acids, containing 20 g l⁻¹ glucose, buffered with 0.165 M MOPS to pH 7.2 and supplemented with 20 mg l⁻¹ l-histidine or l-arginine) to an OD₆₀₀ of 0.2 and incubating for 3 h until they reached the exponential growth phase. All OD measurements were carried out by using a µQuant microtiter plate spectrophotometer (Biotek) and sample volumes of 180 µl in standard 96-well plates. Treatment with fungicides was carried out during the subsequent main culture and appropriate solvent controls were used for comparison.

Susceptibility assays. Sensitivity of C. albicans to antifungal compounds in liquid culture was determined in microtitre plates (180 µl volume). Samples from an exponentially growing pre-culture, at a concentration of 5000 cells ml⁻¹, were incubated in YNB with dilution series of the compounds at 30 °C for 24 h and growth was determined photometrically by measuring the OD₆₀₀. Combination effects of compounds were investigated using the respective compound mixtures. Experiments were conducted in triplicate cultures. Results from three independent experiments are reported here.

In addition, fluconazole sensitivity was evaluated according to the standard recommendations of EUCAST (EUCAST, 2008) using inocula containing different concentrations of fludioxonil.

Analysis of germ tube formation. C. albicans from an exponentially growing pre-culture were incubated in RPMI 1640 (buffered to pH 7 with 0.165 M MOPS) and supplemented with or without 20 µg ml⁻¹ fludioxonil at 37 °C for 4 h. Every 60 min, a sample was analysed microscopically. A total of 300–1500 yeast cells were counted in each experiment and the portion of cells that had formed germ tubes was documented. Data from two independent experiments are presented.

Transcriptome analysis by microarray. C. albicans from an exponentially growing pre-culture were incubated in RPMI 1640 (buffered to pH 7 with 0.165 M MOPS) and supplemented with, or left without, 20 µg ml⁻¹ fludioxonil at 37 °C for 30 min. The cells were harvested by centrifugation at 8000 g for 2 min and the cell pellets were shock-frozen in liquid nitrogen. Frozen pellets were suspended in 0.6 ml RLT buffer (Qiagen) and mechanically disrupted using glass beads (425–600 µm diameter, Sigma). RNA was isolated on RNeasy mini columns with added DNase (Qiagen) as recommended by the manufacturer. The quality and integrity of total RNA of the samples was controlled with an Agilent 2100 Bioanalyzer.

Cy3-labelled cRNA was transcribed using the QuickAmp Labelling kit (Agilent). According to the manufacturer’s recommendations, a One-Colour RNA Spike-In kit (Agilent) was used as spike-in control. Labelled cRNA (600 ng) was hybridized to custom 8 × 15k microarrays from Agilent (GEO platform accession GPL15859), which contained 2–4 probes for 6203 C. albicans genes, as well as 20 probes each for 10 spike-in controls and 336 probes for hybridization and grid controls. The microarrays were scanned on a G2565A scanner (Agilent) and feature extraction and quality control were performed in the Feature Extraction software version 10.7.3.1 (Agilent) using the protocol ‘GE1_107_Sep09’. Microarray design and hybridization, as well as image analysis were performed at the Microarray Core Facility of the Helmholtz Centre for Infection Research, Braunschweig, Germany.

Data from three biologically independent experiments were analysed using the R/Bioconductor software package limma (Gentleman et al., 2004; Smyth, 2004). Between-array-normalization by a quantile-method (Bolstad et al., 2003; Smyth & Speed, 2003) was followed up by finding the mean of the intensities of replicate probes and calculating the logarithmic fold-change and an empirical Bayes moderated t-statistic (Smyth, 2004). Gene annotation data were from the Candida Genome Database (Arnaud et al., 2012; C. albicans_SC5314_version_A21-002-m03-r02_chromosomal_feature.tab.gz). The raw data and results can be accessed at the NCBI Gene Expression Omnibus (accession no. GSE39715).

Gene expression analysis by RT-PCR. For the working culture, the pre-culture was diluted to an OD₆₀₀ of 0.2 in YNB supplemented with, or left without, up to 20 µg ml⁻¹ fludioxonil. After cultivation at 30 °C for 30 min, the cells were harvested, shock-frozen in liquid nitrogen and RNA was isolated, as described above.

Three micrograms of total RNA were employed in reverse transcription, with superscript II RT, random and oligo-dt₁₂₋₁₈ primers (Invitrogen), according to the manufacturer’s recommendations. Quantitative real-time PCR was carried out on a 96-well LightCycler 480 system using the LightCycler 480 SYBR Green I Master (Roche), as recommended by the manufacturer (45 cycles of
95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s). Gene sequences were obtained from the Candida Genome Database (Inglis et al., 2012) and gene-specific oligonucleotides (Table S1, available in JMM Online) were designed by Roche's Probe Library Assay Design Center and synthesized by Eurofins MWG Operon. Specificity was controlled against the C. albicans genome sequence by using BLAST. Real-time analysis data (crossing-points) were normalized with respect to the actin gene ACT1 and relative gene expression levels were calculated. The mean and SD of the gene expression levels relative to solvent controls in three independent experiments were calculated and the significance of the changes in gene expression was tested by using a Student’s t-test of normalized data (P<0.05).

**RESULTS AND DISCUSSION**

**Fludioxonil caused a mild inhibition of growth and germ tube formation in C. albicans SC5314**

We have previously shown that the type III histidine kinase of C. albicans, Nik1p, is the target of fludioxonil (Buschart et al., 2012). However, inhibition of growth of C. albicans by fludioxonil is strongly dependent on the genetic background of the strain (Wesolowski et al., 2010). Growth of C. albicans ATCC-10231 was severely inhibited by concentrations >5 µg ml⁻¹, while growth of C. albicans DSM-1577 and C. albicans SC5314, which is most commonly used in genetic studies, were only affected at high concentrations (Fig. 1a).

It was reported that Nik1p is necessary for a full yeast–hyphae transition (Alex et al., 1998; Yamada-Okabe et al., 1999). For this reason, morphological analyses of C. albicans treated with fludioxonil were conducted. When C. albicans SC5314 was grown under hyphae-inducing conditions, i.e. pH 7 and 37 °C, fludioxonil caused a slight reduction in germ tube formation and growth during the first 4 h of induction (Fig. 1b shows data after 2 h).

**Fludioxonil induced the expression of CDR1 and CDR2 genes**

To further characterize the effects of fludioxonil on C. albicans, changes in the gene expression of C. albicans grown under hyphae-inducing conditions in response to treatment with 20 µg ml⁻¹ fludioxonil were analysed. The presence of fludioxonil led to the differential expression of relatively few genes, with only 32 genes having positive Bayes posterior log odds-ratios of differential expression (Tables S2 and S3). Among the top 20 genes ranked by the significance of their differential expression (Table S2), only 10 genes were annotated with a putative or proven function. In accordance with the relevance of the fludioxonil-target Nik1p for germ tube formation, the gene for a known indicator of hyphal formation, hyphal wall protein 1 (HWPI; Staab et al., 1996; Staab & Sundstrom, 1998), was downregulated under fludioxonil treatment. We also observed the induction of ABC transporter genes CDR1 and CDR2. Below the threshold for significance, the CDR4 gene was also induced, while other important transporter genes, such as MDR1, were not differentially expressed.

Due to the relevance of the ABC transporters Cdr1p and Cdr2p for the resistance to antimycotics, we analysed the expression of the encoding genes under culture conditions favouring yeast growth in more detail. The expression of both CDR1 and CDR2 genes was dependent on the concentration of fludioxonil (Table 1). Concentrations as low as 2.2 µg ml⁻¹ led to an upregulation of CDR1, hence the

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**Fig. 1.** (a) Growth inhibitory effect of fludioxonil is dependent on the strain of C. albicans. Wild-type C. albicans SC5314 (●), C. albicans ATCC-10231 (■) and C. albicans DSM-1577 (◆) were incubated with a dilution series of fludioxonil for 24 h, before growth (displayed as the percentage of untreated control) was determined photometrically. (b) Effect of 20 µg ml⁻¹ fludioxonil (grey bars) on the germ tube formation of C. albicans SC5314 and deletion mutants. White bars represent untreated controls. Data are from microscopic analysis after 2 h of incubation in RPMI 1640 at 37 °C. Fludioxonil caused a strong inhibition of germ tube formation in strains lacking CDR1 gene expression. A total of 300–1500 yeast cells were counted in each experiment (total 750–2200 cells) and the portion of cells that had formed germ tubes was documented. Data are presented as the mean percentage of hyphae observed from two independent experiments.
Table 1. Concentration-dependent effects of fludioxonil on growth, CDR1 and CDR2 gene expression and the susceptibility to fluconazole and R6G

<table>
<thead>
<tr>
<th>Fludioxonil (µg ml⁻¹)</th>
<th>C. albicans DSM 1577</th>
<th>C. albicans SC5314</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ ± sd (µg ml⁻¹)*</td>
<td>IC₅₀ ± sd (µg ml⁻¹)*</td>
</tr>
<tr>
<td></td>
<td>Flunconazole</td>
<td>R6G</td>
</tr>
<tr>
<td>0</td>
<td>0.1 ± 0.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>0.25</td>
<td>0.2 ± 0.2</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>0.74</td>
<td>0.3 ± 0.3</td>
<td>0.7 ± 0.1§</td>
</tr>
<tr>
<td>2.2</td>
<td>0.5 ± 0.4</td>
<td>0.7 ± 0.1§</td>
</tr>
<tr>
<td>6.7</td>
<td>0.5 ± 0.3§</td>
<td>0.7 ± 0.2§</td>
</tr>
<tr>
<td>20</td>
<td>0.6 ± 0.1§</td>
<td>0.9 ± 0.1§</td>
</tr>
</tbody>
</table>

*IC₅₀ values were determined photometrically after growth of culture to 5000 cells ml⁻¹ for 24 h.
†Gene expression as compared to untreated cells was measured by qRT-PCR after 30 min of treatment.
§Reference samples for gene expression.
§P < 0.05.

Active concentrations for this induction were lower than the concentrations that led to a significant growth inhibition.

However, the induction of the ABC transporter genes was independent of the presence of the histidine kinase Nik1p, which is the target of fludioxonil, as induction was also observed in the deletion mutant Δnik1 (Fig. 2). Therefore, the induction of CDR1 and CDR2 is to be considered a target-independent side effect of fludioxonil in C. albicans.

The CDR1 and CDR2 genes were expressed at a basal level similar to the wild-type in a Δtac1 mutant (data not shown). Induction of CDR2 gene expression by fludioxonil was completely dependent on the regulator Tac1p (Fig. 2), while the CDR1 gene was still induced significantly (P < 0.05) by fludioxonil in the absence of Tac1p, although to a lesser extent than in the wild-type. This is in agreement with the earlier finding that Tac1p is not essential for basal Cdr1p-expression (Coste et al., 2004) and that further factors can be involved in the induction of the CDR1 gene (Shukla et al., 2011).

In contrast to the herbicides acetochlor, metolachlor, dimethenamid and glyphosate, which were shown by Schmidt et al. (2008) to induce CDR gene expression at certain concentrations that were also fungidical or fungistatic, fludioxonil led to an induction of the CDR1 gene at subinhibitory concentrations. These active concentrations were in the ranges of allowed (up to 20 µg ml⁻¹), as well as occasionally observed (up to 1 µg ml⁻¹), residual concentrations on fruit (European Commission, 2011; EFR, 2012).

**Aggravation of fludioxonil activity by deletion of the CDR1 gene**

As both ABC transporter genes CDR1 and CDR2 were upregulated in response to fludioxonil, the effect of these transporters on the activity of fludioxonil was investigated using deletion mutants. Growth of C. albicans SC5314 cells was inhibited only slightly and transiently by fludioxonil and similar results were found in CDR1 and CDR2 gene deletion mutants (data not shown). We also tested the effect of fludioxonil on hyphae formation. As shown in Fig. 1b, fludioxonil strongly reduced the ability to form germ tubes in the Δcdr1 and Δcdr1,2 mutants. The Δcdr2 mutant was affected only by a similar degree to the wild-type. This indicated that the fungicide was exported out of the cell by Cdr1p in wild-type cells. While the induction of CDR2 gene expression by fludioxonil was stronger than that of CDR1, the CDR1 gene seemed to play a more important role in this process, as the Δcdr2 mutant behaved similar to the wild-type. This finding is similar to the case of fluconazole, where the CDR1 gene plays the bigger role in resistance (Tsao et al., 2009).

Treatment of a mutant strain carrying a deletion in the gene TAC1, which codes for the known regulator of the CDR1 gene, also caused only a slight reduction in germ tube formation. This was in accordance with the partial induction of CDR1 gene expression in the Δtac1 mutant and the basal expression of the CDR1 gene in this mutant.

**Fludioxonil influenced the export rate of rhodamine 6G**

The potential role of fludioxonil in drug resistance was evaluated by studying the effect of fludioxonil on the export efficiency of the ABC transporters in extrusion of a known cargo, rhodamine 6G (R6G; Maesaki et al., 1999).

R6G (4.8 µg ml⁻¹) was added to de-energized cells and taken up by passive diffusion, as observed via the absorbance of the culture supernatant. The rapid diffusion rate was...
unchanged in the presence of 20 μg ml⁻¹ fludioxonil in comparison to control cultures as the supernatant contained <250 ng ml⁻¹ R6G in each case after 30 min. It was also the same for the wild-type and the Δnik1 mutant.

After transfer into glucose-containing medium, C. albicans was able to produce the ATP needed for the export of R6G by the ABC transporters. Consequently, R6G was exported actively with an extrusion rate higher than the inward diffusion rate, resulting in a net efflux, which was visible from the increase in absorbance of the supernatant. Cultures that were treated with fludioxonil during the efflux phase displayed a decreased R6G efflux rate in comparison to control cultures (Fig. 3a). Fludioxonil added during the diffusion phase of the experiment displayed the same effect as fludioxonil added during the efflux phase of the experiment (data not shown). However, R6G efflux was increased if the pre-cultures had been treated with fludioxonil before loading with R6G (Fig. 3a).

These results can be explained by competition of R6G with fludioxonil for binding sites within the ABC transporters. When fludioxonil and R6G are added simultaneously to C. albicans, only the basal efflux capacity is available for both compounds. This resulted in a decelerated efflux rate for R6G. Pre-incubation of the cells with fludioxonil before starvation and loading with R6G led to the increased expression of the CDR1 gene and, thus, increased efflux capacity. This could be observed after supplying the cells with glucose as an energy source.

The changes in R6G efflux rates in the presence of fludioxonil observed in the wild-type were also detected in the Δnik1 mutant (Fig. 3b), pointing, again, to the fact that this effect is independent of Nik1p.

Growth inhibitory effect of R6G was not significantly altered by fludioxonil

As R6G is toxic to C. albicans and could not be extruded as effectively by C. albicans simultaneously treated with fludioxonil, the effect of fludioxonil on the toxicity of R6G was examined. Only with high concentrations of fludioxonil was a slight increase of the growth inhibitory effect of R6G detected (Table 1). Therefore, the attenuation of R6G export by fludioxonil did not lead to a synergistic effect between the two substances.

Resistance to fluconazole was increased by fludioxonil

Another known cargo of the ABC transporters of C. albicans is the antifungal fluconazole (Sanglard et al., 1995). As the ABC transporters play a vital role in resistance development against this drug, the effect of fludioxonil on the susceptibility to fluconazole was also analysed. Concurrent
exposure of C. albicans SC5314, as well as the fludioxonil-resistant C. albicans DSM-1577, to fludioxonil and fluconazole led to an increase in fluconazole resistance by a factor of ~3 (Table 1). This effect was also observed in a standardized susceptibility assay using the EUCAST method (EUCAST, 2008), where a change of MICs from 0.5 µg ml$^{-1}$ (control) to 4 µg ml$^{-1}$ (treated with 20 µg ml$^{-1}$) was observed. The effect was dependent on the concentration of fludioxonil and the active concentration range corresponded to the concentration range that was sufficient to trigger induction of CDR1 gene expression. Thus, like the induction of CDR1 gene expression, increased resistance to fluconazole was caused by fludioxonil concentrations within the allowed maximum residue levels (European Commission, 2011; EFR, 2012).

While the exposure to fludioxonil led to a decrease in susceptibility to fluconazole, the activity of R6G was slightly enhanced, as described above. This may be explained by an interaction of fluconazole and R6G with different subdomains of the ABC transporters, which was revealed by a recent study of hybrid ABC transporters (Tanabe et al., 2011). Therefore, fludioxonil may compete with R6G for binding sites and the binding site for fludioxonil seems to be more closely related to that of R6G.

### Fludioxonil-induced decrease in fluconazole susceptibility was independent of Nik1p, but CDR1 gene expression was essential for fluconazole resistance

The factors responsible for the increase of resistance to fluconazole due to fludioxonil exposure were further analysed using deletion mutants. An earlier study indicated that the histidine kinase Chk1p and a response regulator, Ssk1p, from C. albicans were involved in resistance to fluconazole, as the uptake of the drug and, thus, the intracellular concentrations of the drug, as well as the susceptibility were increased in knockout mutants of the respective mutants (Chauhan et al., 2007). However, the basal level of susceptibility to fluconazole was similar in the wild-type and the Δnik1 mutant. Moreover, we observed a fludioxonil-triggered increase of fluconazole resistance, which was in accordance with the induction of the CDR1 and CDR2 genes. We assume that this increased the efflux capacity for fluconazole. Both observations were independent of the presence of the histidine kinase Nik1p, which is the known target of fludioxonil (Table 2). The difference of the roles of Nik1p and Chk1p in fluconazole susceptibility is likely due to activation of different signal transduction pathways, which is generally assumed because of phenotypic differences in the respective deletion mutants (Chauhan & Calderone, 2008).

The antagonistic effect of fludioxonil was observed in all analysed deletion mutants with the exception of those carrying deletions in the CDR1 gene (Table 2). The Δcdr1 mutants were, as expected, hypersensitive to fluconazole. The decrease in susceptibility to fluconazole caused by fludioxonil was not dependent on the known activator of CDR1 gene expression, Tac1p. This was in agreement with the finding that the CDR1 gene was expressed and at least partially induced by fludioxonil in a Δtac1 mutant (Fig. 2). As the involvement of further transcription factors in CDR1 gene induction has been shown (Shukla et al., 2011), fludioxonil-triggered CDR1 gene induction is likely to be promoted by another transcription factor in addition to Tac1p.

### CONCLUSION

Exposure of C. albicans to the fungicide fludioxonil triggered a concentration-dependent induction of the expression of ABC transporter genes CDR1 and CDR2, which are involved in resistance to antymycotics such as fluconazole. As a consequence, the resistance of C. albicans to fluconazole increased, so that fludioxonil indirectly antagonized the activity of fluconazole. This induction effect occurred at subgrowth inhibitory concentrations of the fungicide and was also independent of the primary target of fludioxonil in C. albicans, the histidine kinase Nik1p, which is involved in morphological changes as well as in stress response.

### Table 2. Effect of fludioxonil on the susceptibility of C. albicans deletion mutants to fluconazole

<table>
<thead>
<tr>
<th>Fludioxonil (µg ml$^{-1}$)</th>
<th>IC$_{50}$ ± SD of fluconazole* (µg ml$^{-1}$)</th>
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<tr>
<td></td>
<td>SC5314</td>
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<tr>
<td>0</td>
<td>0.3 ± 0.1</td>
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<td>0.25</td>
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<td>0.74</td>
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<td>20</td>
<td>0.9 ± 0.1†</td>
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*IC$_{50}$ values were determined photometrically after growth of culture to a concentration of 5000 cells ml$^{-1}$ for 24 h.

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