

## Editor's Choice

The bactericidal activity of  $\beta$ -lactam antibiotics is increased by metabolizable sugar speciesMette Thorsing,<sup>1</sup> Thomas Bentin,<sup>2</sup> Michael Givskov,<sup>1,3</sup>  
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Here, the influence of metabolizable sugars on the susceptibility of *Escherichia coli* to  $\beta$ -lactam antibiotics was investigated. Notably, monitoring growth and survival of mono- and combination-treated planktonic cultures showed a 1000- to 10 000-fold higher antibacterial efficacy of carbenicillin and cefuroxime in the presence of certain sugars, whereas other metabolites had no effect on  $\beta$ -lactam sensitivity. This effect was unrelated to changes in growth rate. Light microscopy and flow cytometry profiling revealed that bacterial filaments, formed due to  $\beta$ -lactam-mediated inhibition of cell division, rapidly appeared upon  $\beta$ -lactam mono-treatment and remained stable for up to 18 h. The presence of metabolizable sugars in the medium did not change the rate of filamentation, but led to lysis of the filaments within a few hours. No lysis occurred in *E. coli* mutants unable to metabolize the sugars, thus establishing sugar metabolism as an important factor influencing the bactericidal outcome of  $\beta$ -lactam treatment. Interestingly, the effect of sugar on  $\beta$ -lactam susceptibility was suppressed in a strain unable to synthesize the nutrient stress alarmone (p)ppGpp. Here, to the best of our knowledge, we demonstrate for the first time a specific and significant increase in  $\beta$ -lactam sensitivity due to sugar metabolism in planktonic, exponentially growing bacteria, unrelated to general nutrient availability or growth rate. Understanding the mechanisms underlying the nutritional influences on antibiotic sensitivity is likely to reveal new proteins or pathways that can be targeted by novel compounds, adding to the list of pharmacodynamic adjuvants that increase the efficiency and lifespan of conventional antibiotics.

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## INTRODUCTION

The emergence and dissemination of antibiotic resistance is a significant threat to global public health (WHO, 2014). As new antibiotics are rarely discovered (Ling *et al.*, 2015), alternative approaches such as combination therapies constitute a promising strategy to extend the lifespan of existing antibiotics (Ejim *et al.*, 2011). Several decades of antibiotic research have provided insight into the molecular interactions between antimicrobials and their targets, and their consequences for the bacterial cell; however, much remains to be learned about exogenous and endogenous

factors affecting the outcome of antibiotic therapy. Understanding the link(s) between environmental changes and the consequent changes in cell physiology and antibiotic tolerance can provide important insight into the bactericidal activity of antimicrobials as well as mechanisms contributing to antibiotic tolerance, which may be targeted by adjuvants to conventional antibiotics. The penicillin-binding proteins (PBPs) are major determinants of *Escherichia coli* cell morphology and targets of a clinically important class of antibiotics: the  $\beta$ -lactams. The essential PBP3 (FtsI) is required for septum formation during cell division (Chung *et al.*, 2009) and inhibition of PBP3 via  $\beta$ -lactam-mediated blockage leads to the formation of polynucleoid bacterial filaments, which eventually lyse through an unknown mechanism (Spratt, 1975). Bacterial cell division is regulated according to nutrient availability, but the manner in which nutritional status is relayed to the control of the cell division machinery is still poorly understood, although several

Abbreviations: FSC, forward scatter; MBC, minimum bactericidal concentration; PBP, penicillin-binding protein; SPB, sugar potentiation of  $\beta$ -lactam antibiotics; SSC, side scatter.

Five supplementary figures are available with the online Supplementary Material.

metabolites have been suggested to play a role in cell size regulation and cell division (Hill *et al.*, 2013; Monahan *et al.*, 2014). A main regulator of bacterial growth rate and cell division is the nucleotide (p)ppGpp (Potrykus *et al.*, 2011). Two homologous enzymes control the level of (p)ppGpp: RelA, which synthesizes (p)ppGpp under amino acid starvation (Haseltine *et al.*, 1972), and the dual-function synthetase/hydrolase SpoT, which is necessary for (p)ppGpp accumulation during various stresses, including carbon and fatty acid starvation (Seyfzadeh *et al.*, 1993; Xiao *et al.*, 1991). It is well known that high levels of (p)ppGpp give rise to increased  $\beta$ -lactam tolerance due to an indirect inhibitory effect on peptidoglycan biosynthesis (Rodionov & Ishiguro, 1995).

Here, we examined the effect of available carbon sources on *in vitro*  $\beta$ -lactam susceptibility of *E. coli*. We demonstrated that the presence of metabolizable sugars promote lysis of filaments formed following  $\beta$ -lactam exposure, leading to significantly increased bacterial killing, whereas other tested carbon sources had no impact on survival. Furthermore, our results implicated the nutritional alarmone (p)ppGpp in this response, as the advanced lysis was abolished in a (p)ppGpp<sup>0</sup> strain.

## METHODS

**Bacterial strains and growth conditions.** WT *E. coli* K-12 MG1655 or derivatives of MG1655 were used throughout this study [ $\Delta araC$  (Desai & Rao, 2010),  $\Delta glc$  ( $\Delta ptsG$ ,  $\Delta ptsM$ ,  $\Delta glk$  and  $\Delta gcd$ ) (Zuroff *et al.*, 2010), MG1655 (CF1648),  $\Delta relA$  (CF12510) (Brown *et al.*, 2002) and  $\Delta relA\Delta spoT$  (CF10237) (Magnusson *et al.*, 2007)] along with two selected clinical *E. coli* strains, CFT073 (Welch *et al.*, 2002) and IBD p7 (Petersen *et al.*, 2009). All cultures were grown in low-salt Luria–Bertani (LB) broth adjusted to a starting pH of 7.5 at 37 °C with shaking unless otherwise stated. Casamino acids (BD Bacto) were autoclaved separately and added as described in Results. Sterile filtered supplements were added to the medium after autoclaving when required. Ciprofloxacin, gentamicin, cefuroxime and the tested carbon sources were purchased from Sigma-Aldrich, and carbenicillin disodium salt was purchased from Invitrogen.

**Antibiotic susceptibility testing.** For planktonic antibiotic tolerance testing, an overnight culture was diluted 1 : 100 into fresh LB broth and grown until OD<sub>595</sub> 0.48–0.52. Treatment was initiated by aliquoting the culture into a 96-well polystyrene plate containing sugar and antibiotic (240  $\mu$ l cell culture, 10  $\mu$ l antibiotic and 2.5  $\mu$ l sugar). Sugar concentrations were adjusted to deliver 60 mM carbon. Incubation and measurements were carried out in a TECAN Sunrise or F200 microtitre plate reader on living cells at 37 °C every 20 min for a period of 18 h with intermediate shake cycles in linear mode. Cell cultures were allowed to settle for 200 s after each shake cycle before measurement. OD<sub>595</sub> was then measured. Cell survival was estimated after 18 h of treatment by plating appropriate 10-fold serial dilutions of the cultures on LB agar plates followed by determination of the c.f.u. count after overnight growth at 37 °C. RNA polymerase suppressor mutants occur spontaneously at a relatively high frequency in the  $\Delta relA\Delta spoT$  background (Murphy & Cashel, 2003). To measure the frequency of suppressor mutants,  $\Delta relA\Delta spoT$  cultures were plated on M9 (BD Difco) +0.2 % glucose agar plates supplemented with 1  $\mu$ g thiamine ml<sup>-1</sup> after treatment and the percentage revertants was determined as the ratio of c.f.u. ml<sup>-1</sup> on minimal and LB plates. For the data presented here, <5 and <1 % of the population

reverted after growth without or with carbenicillin, respectively. In the figures, error bars indicate SD of *n* biological replicates, where *n* ≥ 3, as indicated in the legends. *P* values were determined by Student's *t*-test using Prism version 5.0.

Minimum bactericidal concentrations (MBCs) for carbenicillin were determined by the broth microdilution method using an overnight culture grown in LB medium without additives. The culture was diluted in fresh LB medium and inocula with cell densities ranging from the standard inoculum of 5 × 10<sup>5</sup> to 5 × 10<sup>8</sup> c.f.u. ml<sup>-1</sup> were grown for 24 h at 37 °C with a twofold serial dilution of carbenicillin ranging from 4 to 1024 mg l<sup>-1</sup> in the presence or absence of a fixed concentration of sugar (corresponding to 60 mM carbon). Two aliquots of 10  $\mu$ l from wells with no visible growth were spotted on LB agar and incubated overnight at 37 °C. MBC was scored as the lowest concentration of carbenicillin from which no viable cells were recovered.

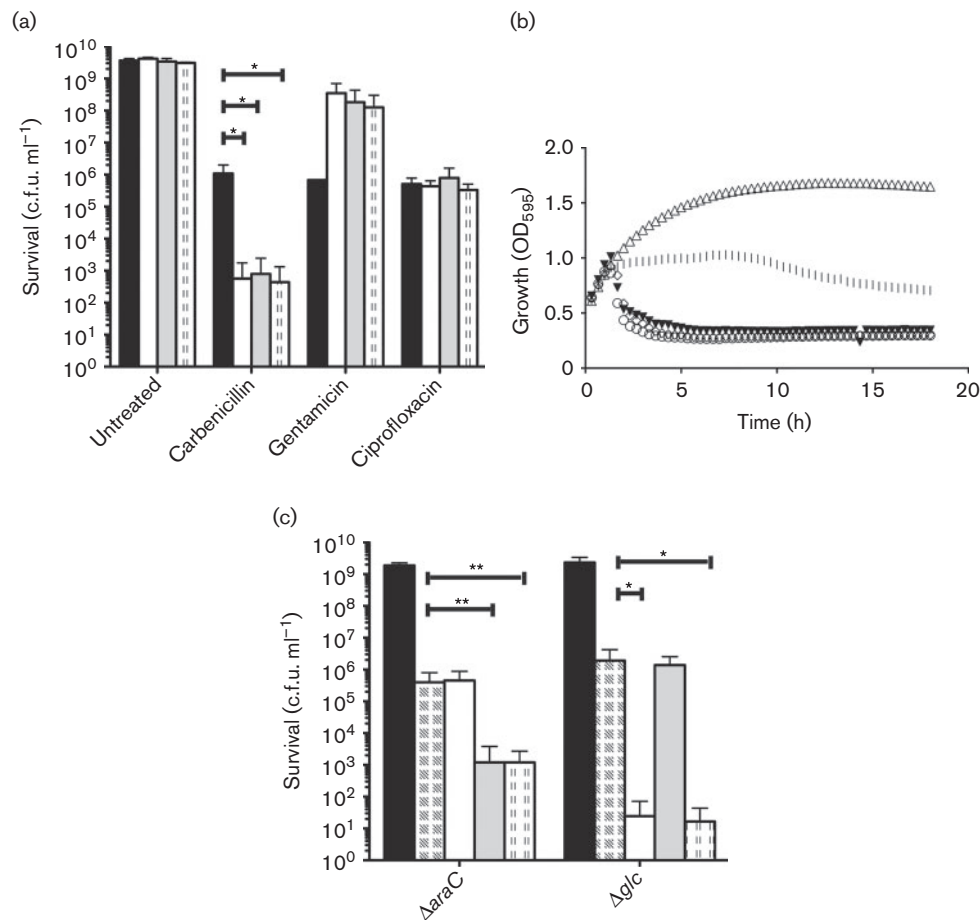
**Flow cytometry.** To characterize the morphology of  $\beta$ -lactam-treated cells, *E. coli* cells were collected at different time points during  $\beta$ -lactam treatment. Approximately 10<sup>6</sup> cells were harvested, resuspended in PBS and profiled on an Accuri C6 flow cytometer counting 50 000 cells per profile. To eliminate non-bacterial particulate material, we used 0.22  $\mu$ m filtered PBS and gated the instrument using an FSC-H threshold of 10 000.

**Microscopy.** *E. coli* cells were collected by centrifugation at 3000 *g* after 18 h of treatment, washed in 0.9 % NaCl and resuspended in a smaller volume of saline. Cells were observed by bright-field microscopy on a thin layer of 1 % (v/w) agarose in 0.9 % NaCl using a Zeiss LSM 710 microscope (Carl Zeiss) equipped with a × 100/1.4 oil objective lens.

## RESULTS

### Sugar metabolites potentiate $\beta$ -lactam action

A prior observation from our lab demonstrating the effect of arabinose on *E. coli* gentamicin susceptibility (Goltermann *et al.*, 2013) led us to test how sugar metabolites impact on *E. coli* susceptibility to antibiotics belonging to the  $\beta$ -lactam, aminoglycoside, and fluoroquinolone classes. Planktonic cultures of *E. coli* in the late exponential growth phase ( $\sim 10^8$  c.f.u. ml<sup>-1</sup>) were treated with 30  $\mu$ g carbenicillin ml<sup>-1</sup>, 3  $\mu$ g gentamicin ml<sup>-1</sup> or 0.125  $\mu$ g ciprofloxacin ml<sup>-1</sup>, giving rise to a 100-fold reduction in viable cell count. Interestingly, the presence of L-arabinose, D-glucose or D-mannitol sensitized *E. coli* to carbenicillin and further reduced survival three to four orders of magnitude following 18 h of treatment (Fig. 1a); from the growth curves it was evident that the effect occurred within the first few hours of treatment, with a sudden drop in culture turbidity indicative of cell lysis (Fig. 1b). Under these *in vitro* conditions, the bactericidal effect of 30  $\mu$ g carbenicillin ml<sup>-1</sup> and 10 mM D-mannitol was similar to that of 60  $\mu$ g carbenicillin ml<sup>-1</sup> in mono-treatment (Fig. S1a, available in the online Supplementary Material). The potentiating effect of D-mannitol was also observed when applied in combination with the cephalosporin cefuroxime (Fig. S1b), indicating that the observed effect on survival applies to different subgroups within the  $\beta$ -lactam class of antibiotics. In the remaining text, we have used the acronym SPB (sugar potentiation of  $\beta$ -lactam antibiotics) to describe the cooperative action between sugar metabolites and  $\beta$ -lactam



**Fig. 1.** Impact of sugar metabolites on *E. coli* antibiotic sensitivity. (a) MG1655 WT was treated with 30  $\mu\text{g}$  carbenicillin  $\text{ml}^{-1}$ , 3  $\mu\text{g}$  gentamicin  $\text{ml}^{-1}$  or 0.125  $\mu\text{g}$  ciprofloxacin  $\text{ml}^{-1}$  alone (black bars) or in combination with 12 mM L-arabinose (white bars), 10 mM D-glucose (grey bars) or 10 mM D-mannitol (dotted bars). Survival was monitored after 18 h of treatment. (b) OD<sub>595</sub> of MG1655 cultures during 18 h of treatment without (open triangles) or with 30  $\mu\text{g}$  carbenicillin  $\text{ml}^{-1}$  alone (vertical lines) or in combination with sugars (arabinose, open circles; glucose, closed triangles; mannitol, open diamonds). (c) Survival of MG1655 mutants deficient in arabinose metabolism ( $\Delta araC$ ) and glucose metabolism ( $\Delta glc$ ) after 18 h of no treatment (black bars), carbenicillin treatment alone (chequered bars) or in combination with sugars (arabinose, white bars; glucose, grey bars; mannitol, dotted bars). Concentrations as in (a). All c.f.u.  $\text{ml}^{-1}$  values are shown as mean  $\pm$  SD ( $n > 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ .

antibiotics. In contrast to what was found for the  $\beta$ -lactam antibiotics, exponential-phase *E. coli* was less sensitive to the bactericidal activity of gentamicin in the presence of sugar, whereas the ciprofloxacin susceptibility of *E. coli* was unaffected (Fig. 1a). *E. coli* cultures treated with different sugars alone showed no sugar-dependent reduction in cell survival (Fig. 1a) and, importantly, addition of sugars did not significantly change the growth rate as judged by OD<sub>595</sub> measurements (Fig. S2). Any changes of growth rate did not correlate with the ability of the sugar to potentiate  $\beta$ -lactam action (Fig. S2).

We then asked if the SPB effects observed using carbenicillin and cefuroxime were general for cell-wall-targeting antibiotics (Fig. S3). An SPB effect was observed for 20  $\mu\text{g}$  D-cycloserine  $\text{ml}^{-1}$  (Fig. S3a, b), which works upstream of carbenicillin

by inhibiting D-Ala-D-Ala formation (Neuhaus & Lynch, 1964). No SPB effect on growth was observed for the penicillin derivative mecillinam (Fig. S3c, d), which targets PBP2 (Spratt, 1975) or for cephalexin (Fig. S3e), a first-generation cephalosporin known to be less efficient against Gram-negatives than second-generation cephalosporins such as cefuroxime.

In addition, we investigated whether the SPB effect also manifests in clinical isolates of *E. coli*, i.e. the uropathogenic CFT073 strain (Welch *et al.*, 2002) and the inflammatory bowel disease isolate IBD p7 (Petersen *et al.*, 2009) (Fig. S4). Similar to MG1655, the CFT073 strain and the IBD p7 strain both displayed a pronounced SPB effect with two to three orders of magnitude reduction in cell survival upon combination treatment using carbenicillin and sugar (Fig. S4).

**Table 1.** Dependency of carbenicillin MBC on inoculum density and presence of sugars

Sugar	MBC ( $\mu\text{g ml}^{-1}$ ) at inoculum density (c.f.u. $\text{ml}^{-1}$ )			
	$5 \times 10^5$	$5 \times 10^6$	$5 \times 10^7$	$5 \times 10^8$
No sugar	16	16	32	256
12 mM L-arabinose	16	16	16	64
10 mM D-glucose	16	16	16	64
10 mM D-mannitol	16	16	16	64

To examine whether  $\beta$ -lactam sensitivity in *E. coli* MG1655 extends to other metabolites, we tested carbenicillin in combination with a selection of carbon sources entering central metabolism via glycolysis, the pentose phosphate pathway, the Entner–Doudoroff pathway, or the tricarboxylic acid cycle. L-Arabinose, glucose, mannitol, xylose, ribose, lactose and galacturonate all increased  $\beta$ -lactam sensitivity. In contrast, glycerol and succinate, which are relatively poor energy/carbon sources, and D-arabinose and sucrose, which cannot be metabolized by *E. coli* MG1655, had no effect on carbenicillin tolerance (Fig. S1c). These results furthermore rule out a change in medium osmolarity as the underlying cause of SPB.

The sensitivity of *E. coli* MG1655 to carbenicillin displayed a pronounced inoculum effect (Table 1), i.e. the antimicrobial efficacy of carbenicillin decreased with increasing bacterial density. Notably, the carbenicillin MBC of a standard inoculum ( $5 \times 10^5$  c.f.u.  $\text{ml}^{-1}$ ) was unaffected by the presence of sugar in the growth medium, whereas the MBC was lowered two- to fourfold at cell densities  $\geq 5 \times 10^7$  c.f.u.  $\text{ml}^{-1}$  (Table 1). Thus, the SPB effect strongly depended on bacterial cell density, with increasing activity at elevated bacterial density.

### Active metabolism is required for $\beta$ -lactam potentiation

We next investigated whether functional sugar metabolism was required for SPB by using an *E. coli* strain deficient in arabinose metabolism through mutation of the transcriptional activator encoded by *araC*. Similar to the WT, the  $\Delta araC$  strain was sensitized to carbenicillin by D-glucose and D-mannitol; however, the survival was unaffected by addition of L-arabinose (Fig. 1c). Correspondingly, a  $\Delta glc$  strain deficient in glucose metabolism was sensitized by the presence of L-arabinose or D-mannitol, but showed no SPB effect upon addition of D-glucose in combination with carbenicillin. These results confirmed that sugar metabolism is essential and, as such, only sugars that were metabolized by the respective strains were able to potentiate the effect of  $\beta$ -lactams (Fig. 1).

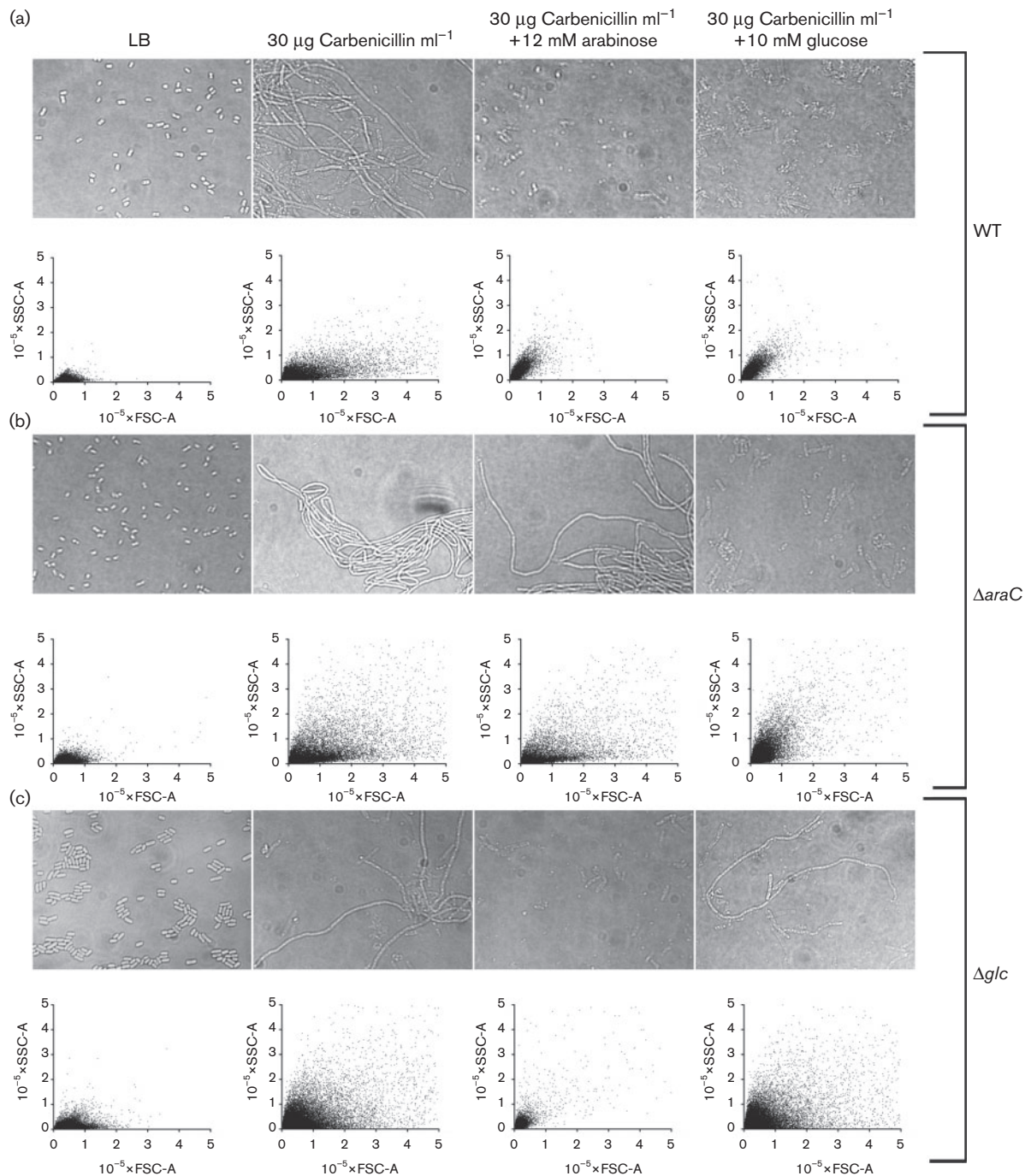
### Sugar metabolites induce morphological changes in $\beta$ -lactam-treated cells

Morphological characterization of carbenicillin-treated cells showed the formation of filaments caused by  $\beta$ -lactam-mediated inhibition of cell division as expected (Fig. 2) (Spratt, 1975). These filaments were, however, absent from samples treated for 18 h with carbenicillin and sugar in combination (Fig. 2), and only semi-transparent cell debris remained. Flow cytometry confirmed the microscopy results and showed a wide distribution of filament sizes (as measured by forward scatter (FSC)) and granularity [as measured by side scatter (SSC)] in the carbenicillin-treated population whereas only small granular debris remained after 18 h combination treatment with carbenicillin and sugar (Fig. 2). By comparison, untreated bacteria consisted of a population of small, smooth cells characterized by low FSC and SSC values grouping to the lower left corner of the flow cytometry diagram. Following treatment with carbenicillin, filaments formed, as detected by an increase in the FSC values stretching along the horizontal axis. As the filaments lysed, when carbenicillin was combined with sugar, the FSC values decreased. Time-course flow cytometry showed that bacterial filaments rapidly formed upon  $\beta$ -lactam mono-treatment and accumulated throughout the treatment period. The presence of metabolizable sugars in the medium did not change the initial rate of filamentation, but led to lysis of the filaments, as indicated by a significant change in the flow cytometry profile between 2 and 4 h of treatment (Fig. S5). The morphological changes in the combination-treated cultures were dependent on active sugar metabolism. The metabolism-deficient  $\Delta araC$  and  $\Delta glc$  strains were morphologically unaffected by addition of arabinose and glucose, respectively, whilst filaments were still eradicated upon addition of metabolizable sugar (Fig. 2).

### Bacterial stringent response modulates the SPB effect

At high cell densities, when nutrients are limited, the level of intrabacterial (p)ppGpp increases, thereby aiding the adaptation of bacteria to suboptimal growth conditions (Potrykus *et al.*, 2011). High levels of (p)ppGpp can cause  $\beta$ -lactam tolerance due to an indirect inhibitory effect on peptidoglycan synthesis (Rodionov & Ishiguro, 1995). We therefore speculated that dense bacterial cultures might accumulate (p)ppGpp due to exhaustion of the preferred carbon sources (Sezonov *et al.*, 2007), and that addition of metabolizable sugars would decrease the (p)ppGpp level and consequently abolish  $\beta$ -lactam tolerance. To test this hypothesis,  $\Delta relA$  and  $\Delta relA \Delta spoT$  mutants and their isogenic parent were treated with carbenicillin in combination with sugar as described above. Following combination treatment, survival of the  $\Delta relA$  strain was reduced approximately three orders of magnitude (Fig. 3), which is comparable with the isogenic

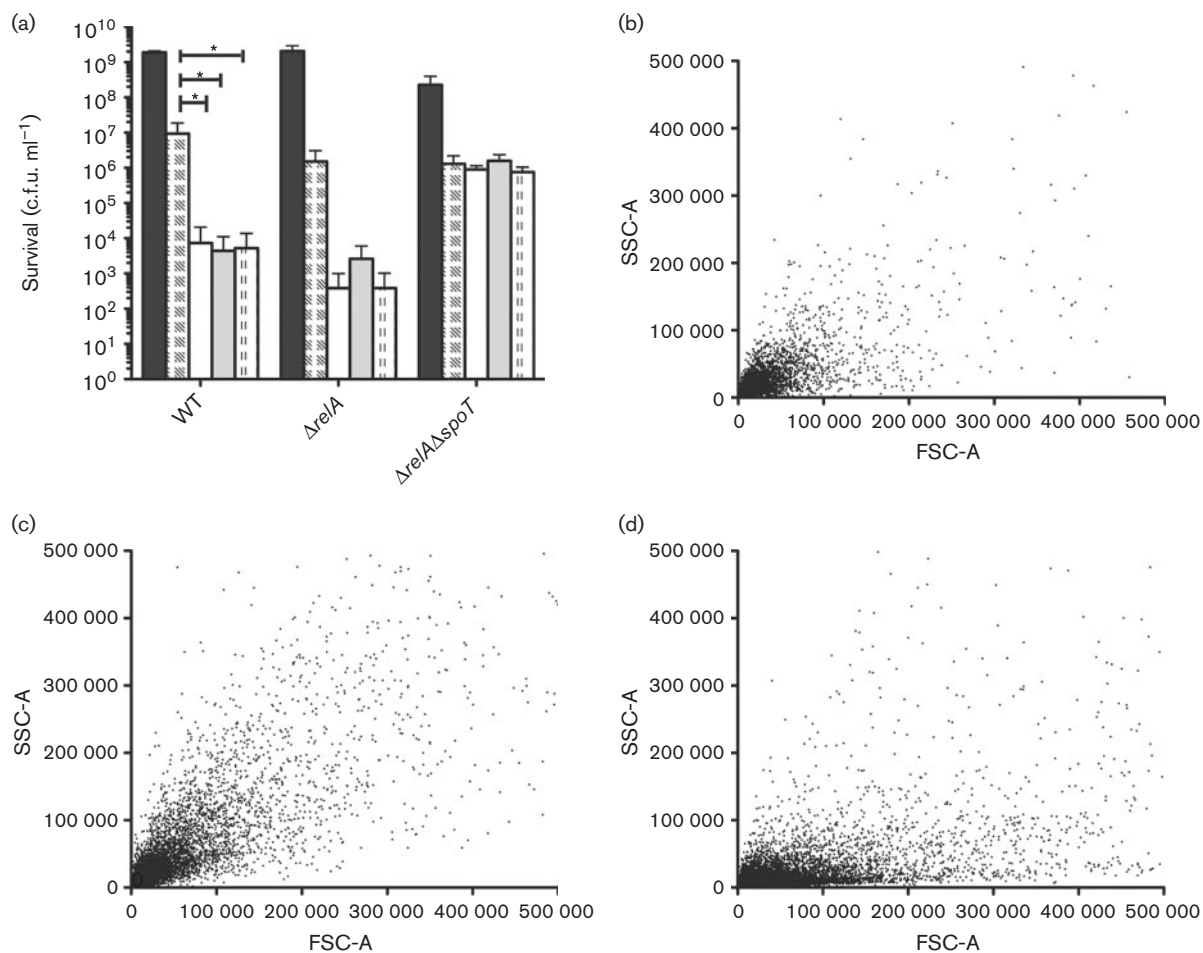




**Fig. 2.** Filament formation and lysis of *E. coli* MG1655 WT (a) and mutants deficient in arabinose metabolism ( $\Delta araC$ ) (b) or glucose metabolism ( $\Delta glc$ ) (c) were treated with carbenicillin alone or in combination with 12 mM L-arabinose or 10 mM D-glucose. Untreated controls were included. Cells were analysed by light microscopy (upper panel) and flow cytometry (lower panel) after 18 h of treatment. Flow cytometry results are shown as FSC-A, indicating cell size, versus SSC-A, indicating surface granularity. Scale bar 10 microns.

WT. However, the potentiation was completely lost in the  $\Delta relA \Delta spoT$  background (Fig. 3), suggesting that the (p)ppGpp level or the SpoT protein itself is important for the SPB effect. In concordance, flow cytometry data

indicated that bacterial filaments formed by the WT and  $\Delta relA$  strains were eradicated (Fig. 3b, c), whereas the  $\Delta relA \Delta spoT$  strain remained filamentous after treatment with carbenicillin in combination with glucose (Fig. 3c).



**Fig. 3.**  $\beta$ -Lactam sensitivity of a (p)ppGpp<sup>0</sup> strain is unaffected by sugar. (a) Bacterial cell survival was determined after 18 h of treatment without (black bars) or with 30  $\mu$ g carbenicillin ml<sup>-1</sup> alone (chequered bars) or in combination with 12 mM L-arabinose (white bars), 10 mM D-glucose (grey bars) or 10 mM D-mannitol (dotted bars) for WT,  $\Delta$ relA and  $\Delta$ relA $\Delta$ spoT strains producing decreasing amounts of the (p)ppGpp alarmone. Data shown as mean  $\pm$  SD ( $n \geq 4$ ). \* $p < 0.05$ . (b–d) Flow cytometry FSC versus SSC profiles of WT (b),  $\Delta$ relA (c) and  $\Delta$ relA $\Delta$ spoT (d) strains after 18 h of treatment with 30  $\mu$ g carbenicillin ml<sup>-1</sup> in combination with 10 mM D-glucose.

As a control, we treated WT cultures in the late exponential growth phase with carbenicillin in combination with individual amino acids (glycine, L-serine, L-alanine or L-threonine), which are among the amino acids depleted first from complex media (Prüss *et al.*, 1994; Sezonov *et al.*, 2007), or with 0.2 % Casamino acids and determined survival following 18 h of incubation. None of these supplements affected survival during carbenicillin treatment (Fig. S1d), confirming that the SPB effect on  $\beta$ -lactam antibiotics was specific to the metabolizable sugars and not a general effect of delaying nutrient depletion of the growth medium.

## DISCUSSION

Here, we report the novel observation that planktonic *E. coli* was sensitized to  $\beta$ -lactam antibiotics when

a metabolizable sugar (e.g. L-arabinose, D-glucose or D-mannitol) was administered along with the antibiotic – an effect which we have dubbed ‘sugar potentiation of  $\beta$ -lactam antibiotics (SPB)’. The cooperative action of sugar and  $\beta$ -lactam led to a 1000- to 10 000-fold reduction in bacterial survival compared with the  $\beta$ -lactam alone in a manner specific to this combination. Sugar was unable to potentiate antimicrobials targeting other cellular functions (DNA replication and translation) and, conversely,  $\beta$ -lactam susceptibility was unaffected by non-carbohydrate carbon sources. If the observed effect was simply a result of increased growth rate, we would expect a similar potentiation of aminoglycoside and quinolone antibiotics as susceptibility to both increases with growth rate (Eng *et al.*, 1991). Moreover, other carbon sources such as Casamino acids did not potentiate the effect of carbenicillin, although these would also be

expected to increase general metabolism in late exponential growth phase. The addition of excess glucose or other sugars could furthermore be expected to result in increased acetate production (Rice *et al.*, 2005), which in *Staphylococcus aureus* has been linked to increased  $\beta$ -lactam susceptibility (Rice *et al.*, 2005). However, a similar link has not been demonstrated for Gram-negative bacteria and as our results could be reproduced in *E. coli* BL21(DE3) (data not shown), a strain which is known to have low acetic acid production (Bäcklund *et al.*, 2011), we do not expect overflow metabolism to be part of the underlying mechanism. Rather, we believe that  $\beta$ -lactam potentiation relies on interplay between specific metabolites, their effect on cell division and the inhibitory effect of  $\beta$ -lactams on PBP function.

The  $\beta$ -lactams used in this study (carbenicillin and cefuroxime) have relatively high affinities for PBP3 and PBP1a (Curtis *et al.*, 1979), and  $\beta$ -lactam treatment leads to formation of polynucleoid filaments due to PBP3 inhibition at sub-MIC concentrations. Similar filamentation was observed when sugar and carbenicillin were combined; however, as indicated by culture turbidity and flow cytometry (FSC), the filaments rapidly lysed after  $\sim 2$ –4 h of treatment. Using metabolism-deficient mutants, we confirmed that active sugar metabolism was necessary for the SPB effect. Rapid  $\beta$ -lactam-induced lysis is often preceded by the formation of membrane protrusions emanating from the mid-cell (Staugaard *et al.*, 1976; Yao *et al.*, 2012), which indicates that bacteria are particularly prone to  $\beta$ -lactams during septation. The cell division is governed by a protein complex, the divisome, which forms at the mid-cell beginning with polymerization of FtsZ into a contractile ring. In *E. coli*,  $\beta$ -lactam antibiotics activate the SOS response leading to controlled inhibition of FtsZ polymerization and temporary  $\beta$ -lactam tolerance (Miller *et al.*, 2004). Cell division is furthermore regulated according to nutrient availability in a manner that is not fully understood, although several modulators of FtsZ polymerization have been found to link cell division with central carbon metabolism in *E. coli* and *Bacillus subtilis* (Hill *et al.*, 2013; Monahan *et al.*, 2014; Weart *et al.*, 2007). The metabolizable sugars tested in the present study quickly promote  $\beta$ -lactam-induced lysis of *E. coli*; thus, we speculate that the nutritional up-shift leads to changes in the divisome that either advance cell division leading to recruitment of murein hydrolases or impede the implementation of protective measurements such as controlled arrest of cell division that could otherwise avert lysis. This notion is supported by the observation that neither mecillinam nor cephalixin was potentiated by sugar metabolites under our experimental conditions. Although both of these  $\beta$ -lactams give rise to morphological alterations (round cells and filaments, respectively), they are non-lytic and non-lethal at high cell densities (Aono *et al.*, 1979; Chung *et al.*, 2009), suggesting that sugar metabolites do not destabilize bacterial filaments

*per se*, but rather reinforce the existing lytic effect of  $\beta$ -lactam antibiotics.

Notably, the effect of sugar on  $\beta$ -lactam sensitivity was completely lost in a  $\Delta relA\Delta spoT$  mutant, which was unable to synthesize (p)ppGpp, as opposed to the WT and  $\Delta relA$  strains. High levels of (p)ppGpp have been linked to antibiotic tolerance in *E. coli* and several other organisms (Abranches *et al.*, 2009; Geiger *et al.*, 2014; Pomares *et al.*, 2008; Rodionov & Ishiguro, 1995), and SpoT is responsible for (p)ppGpp accumulation in response to various environmental cues, including carbon limitation (Xiao *et al.*, 1991). However, because  $\beta$ -lactam sensitivity was unaffected when amino acids were added as a carbon source, we find it unlikely that the SPB effect is caused solely by a SpoT-mediated reduction in (p)ppGpp levels in response to the availability of carbon. However, we cannot exclude that sugars specifically regulate the enzymic activities of SpoT in a manner that is unrelated to total carbon accessibility. In this regard, it will be interesting to know whether carbohydrate and non-carbohydrate carbon sources exert different regulatory effects on SpoT activity. Contrary to the inhibitory effect of high (p)ppGpp concentrations on cell division, a basal level of (p)ppGpp is required for cell division to proceed as seen by septation deficiency in the  $\Delta relA\Delta spoT$  mutant leading to filamentation due to unknown mechanisms (Xiao *et al.*, 1991). As an alternative, we speculate that loss of SPB in the  $\Delta relA\Delta spoT$  background can be explained by the inherent septation deficiency of the strain, which possibly masks a nutrient-dependent change in the divisome that could form the basis of potentiation as discussed above.

Early observations have furthermore suggested a link between the second messenger, cAMP, and the regulation of cell cycle progression and envelope biogenesis (Jaffé *et al.*, 1986; Kumar, 1976; Utsumi *et al.*, 1989). The intracellular cAMP level is regulated in response to carbon availability and, based on the observations of Epstein *et al.* (1975), carbon sources supporting SPB under our conditions are expected to give rise to low cAMP levels, whereas succinate, glycerol or Casamino acids lead to higher cAMP levels. Mutants devoid of cAMP (*cya*<sup>-</sup>) are highly resistant to mecillinam (Aono *et al.*, 1979; D'Ari *et al.*, 1988), which can be reversed by addition of cAMP to the growth medium, but only a modest decrease in susceptibility to other  $\beta$ -lactams has been observed (Aono *et al.*, 1979; Jaffé *et al.*, 1983). Any involvement of cAMP as the underlying cause of SPB would imply that low cAMP levels under our conditions sensitize the bacteria to  $\beta$ -lactam antibiotics, which is contrary to previous observations. Furthermore, the addition of 2 mM cAMP to the growth medium did not prevent the SPB effect (data not shown) and, consequently, we find the involvement of cAMP unlikely.

Our findings that survival during gentamicin exposure was improved by the presence of sugar metabolites is in sharp contrast to recent reports demonstrating the ability of

specific carbohydrates to restore the aminoglycoside susceptibility of non- or slowly growing persister cells in planktonic cultures and in biofilms (Allison *et al.*, 2011; Barraud *et al.*, 2013). The authors attributed the effect to the generation of a proton motive force across the cytoplasmic membrane, which facilitates the uptake of aminoglycoside antibiotics in bacteria with otherwise low metabolic activity. In our experimental setup, we examined actively growing bacteria, which have a fundamentally different metabolism and physiology from persisters (Prax & Bertram, 2014). As persisters typically comprise <0.01% of the total cell count in growing cultures (Keren *et al.*, 2004), it is of utmost importance to understand how metabolites affect the antibiotic tolerance of the entire bacterial population. Recent work has linked (p)ppGpp levels with toxin/antitoxin systems and the Lon protease in the context of persister formation (Maisonneuve *et al.*, 2013). It would be interesting to explore these links in our exponential cultures.

In a previous report, Zuroff *et al.* (2010) demonstrated that glucose impacts the  $\beta$ -lactam tolerance of *E. coli* growing in colony biofilms, but failed to detect an effect on planktonic cells as we report here. We believe that this discrepancy is due to differences in  $\beta$ -lactam concentration and/or differences in bacterial growth phase at the onset of antibiotic treatment. As bacteria living in biofilms can be substantially more tolerant to antibiotics than their planktonic counterparts (Stewart & Costerton, 2001), the possibility that metabolites can also sensitize sessile bacteria to  $\beta$ -lactam antibiotics is very intriguing and warrants further investigation.

The SPB effect is an example of altering the phenotypic tolerance of a bacterial population and uncovering the molecular mechanisms underlying SPB will help to provide important insight into this elusive phenomenon as well as the bactericidal effect of  $\beta$ -lactams in general. Our observations, which extend to two different clinical *E. coli* isolates, emphasize the need for a more comprehensive understanding of the link between central carbon metabolism and antibiotic susceptibility, which may add further targets for novel pharmacodynamic adjuvants to our conventional antibiotics.

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