Differences in strategies to combat osmotic stress in Burkholderia cenocepacia elucidated by NMR-based metabolic profiling

Differences in strategies to combat osmotic stress in *Burkholderia cenocepacia* elucidated by NMR-based metabolic profiling

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Running title: B. cenocepacia osmotic stress tolerance

Abstract

Aims: To investigate mechanisms of osmotic tolerance in *Burkholderia cenocepacia,* a member of the *B. cepacia* complex (Bcc) of closely related strains, which is of clinical as well as environmental importance.

Methods and Results: We employed NMR-based metabolic profiling (metabolomics) to elucidate the metabolic consequences of high osmotic stress for five isolates of *B. cenocepacia*. The strains differed significantly in their levels of osmotic stress tolerance, and we identified three different sets of metabolic responses with the strains least impacted by osmotic stress exhibiting higher levels of the osmo-protective metabolites glycine-betaine and / or trehalose. Strains either increased concentrations or had constitutively high levels of these metabolites.

Conclusions: Even within the small set of *B. cenococepacia* isolates, there was a surprising degree of variability in the metabolic responses to osmotic stress.

Significance and impact of the study: The metabolic responses, and hence osmotic stress tolerance, varies between different *B. cenocepacia* isolates. This study provides a first look into the potentially highly diverse physiology of closely related strains of one species of the Bcc and illustrates that physiological or clinically relevant phenotypes are unlikely to be inferable from genetic relatedness within this species group.

Introduction

Cystic fibrosis (CF) is a monogenic multisystem disorder that renders patients immuno-compromised and predisposes them to fungal and bacterial lung infections (Davies et al. 2007). Opportunistic pathogens like *Pseudomonas aeruginosa* establish long-term infections that can last for decades, lead to a downward spiral of inflammation and loss of lung function and are the major cause of morbidity and mortality today (Davies 2002; Davis 2006). In addition to *P. aeruginosa*, infections with methicillin-resistant *Staphylococcus aureus* or a member of the *Burkholderia cepacia* complex (Bcc) are also a great cause of concern. The latter can lead to cepacia syndrome, a rapid deterioration of lung function associated with septicaemia and early patient death (Mahenthiralingam et al. 2002; Davis 2006).

The Bcc is a closely related species group with at least 17 species (Coenye and Vandamme 2003; Vanlaere et al. 2008; Vanlaere et al. 2009). The taxonomy is complex and ecological diversity is high, with ecological significance ranging from plant growth promoting agent to known plant pathogen (Mahenthiralingam et al. 2005). Two species of the Bcc, *B. cenocepacia* and *B. multivorans,* account for more than 85 % of all Bcc isolates recovered from the CF lung. While *B. multivorans* is has recently supplanted *B. cenocepacia* as the most commonly isolated Bcc species in most of the world, *B. cenocepacia* is still of high clinical importance (Govan et al. 2007; LiPuma 2010).

The close genetic proximity of Bcc isolates renders traditional means of species assignment problematic and approaches based on 'housekeeping genes' were developed to provide a quick way to identify Bcc isolates to strain level. Multi-locus sequence typing (MLST) of seven house-keeping genes has become an accepted technique to

identify Bcc members (Baldwin et al. 2005). For *B. cenocepacia*, the species is divided into subgroups based on the sequence of the DNA recombinase gene *recA* (III-A to III-E) (Mahenthiralingam et al. 2000; Baldwin et al. 2005). There are several epidemic lineages within *B. cenocepacia*, most prominently the ET-12 lineage, which falls into the *recA* III-A subgroup (Mahenthiralingam et al. 2005). Strains of these lineages are highly transmissible and cause patient-to-patient spread (Mahenthiralingam et al. 2005; Drevinek and Mahenthiralingam 2010). While it is not completely understood which factors enable ET-12 strains to cause infections in the CF lung, the genome of ET-12 strain J2315 contains an unusually high fraction of pathogenicity islands (Holden et al. 2009; Drevinek and Mahenthiralingam 2010).

Outside the CF lung, *B. cenocepacia* strains have been isolated from the rhizosphere of wheat and maize (Coenye and Vandamme 2003), where they have been shown to act as protection against fungal infection and plant growth promoting agents (Bevivino et al. 2005).

Survival in both environments depends on maintenance of a stable population size and resistance to or at least some sort of tolerance of abiotic and biotic stress. Osmotic stress could potentially be a factor in both the lungs of CF patients (Smith et al., 1996) as well as the rhizosphere. We investigated the osmotic stress tolerance of five *B. cenocepacia* isolates and elucidated the metabolic changes associated with osmotic stress using nuclear magnetic resonance (NMR)-based metabolic profiling. NMR spectroscopy has the advantage of near universal detection, but suffers from relatively low detection thresholds (Grivet et al. 2003). Because osmo-protectants have to be present in the cell in relatively high concentrations and are not restricted to one particular compound class, NMR spectroscopy is ideally suited for measuring metabolic changes in response to high osmolarity and has been successfully used previously to characterise osmotic

stress-induced metabolic phenotypes (Amin et al. 1995; Behrends et al. 2010; Dai et al. 2010; Gavaghan et al. 2010).

We found that within the five investigated *B. cenocepacia* and even within the two ET-12 strains, several metabolic strategies exist to counteract osmotic stress. At high salt levels, strains that accumulated the classic osmo-protectants trehalose and glycine-betaine were less impacted by osmotic stress than strains that increased the intracellular concentration of certain amino acids.

Methods

Bacterial strains

The following *B. cenocepacia* strains were obtained from the BCCM library (Ghent, Belgium) and maintained as glycerol stocks at -80 °C: III-A lineage (and ET-12 lineage): J2315 (LMG 16656/ST28), K56-2 (LMG 18863/ST30), III-B lineage: J415 (LMG 16654; ST34), C1394 (LMG 16659; ST35), and CEP0511 (LMG 18830; ST39); sequence types (ST) were taken from Baldwin *et al*. (2005). For pre-cultures, the bacteria were inoculated in lysogeny broth (LB) (tryptone, 10 g l^{-1}, yeast extract, 5 g l^{-1}, and NaCl, 5 g l^{-1}) from fresh plates and grown for 16 h. The strains were inoculated to identical starting optical densities from pre-culture into 25 ml universal tubes either containing 10 ml LB supplemented with 0.5 mol l^{-1} NaCl (29.2 g l^{-1}) or 10 ml LB supplemented with an equal volume of water (unsupplemented LB). Cultures were grown at 37 °C for 24 h at 200 rpm orbital shaking.

Metabolite analysis

To measure the strains' media utilisation, 1 ml of the culture was sampled, the optical density was recorded and 750 µl of cell-free supernatant were mixed with 200 µl ^2H_2O

containing 5 mmol l^{-1} trimethylsilyl-1-propionic acid-d_4 (TSP), 25 mol l^{-1} sodium azide and 50 µl of 1 mol l^{-1} phosphate buffer, pH 7. The ^2H_2O provided a field frequency lock for the spectrometer and the TSP served as an internal chemical shift reference. To quantify the endo-metabolome (i.e. the intracellular metabolites), the sample was centrifuged for 5 min (RT, 3600 x g), the pellet washed with 5 ml quarter strength Ringer's solution and centrifuged again. Pellets were resuspended in 5 ml MeOH-water 75:25 % (v/v, -20 °C), sonicated for 10 min and vacuum dried. Finally, the pellets were resuspended in 0.6 ml 0.1 mol l^{-1} phosphate, pH 7.0, 90 % D_2O with 1 mmol l^{-1} TSP. Spectra were acquired on a Bruker Avance DRX600 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany), with a magnetic field strength of 14.1 T and resulting ^1H resonance frequency of 600 MHz, equipped with a 5 mm inverse flow probe. Samples were introduced using a Gilson flow-injection autosampler. Spectra were acquired following the approach given in (Beckonert et al. 2007). Briefly, a one-dimensional NOESY pulse sequence was used for water suppression; data were acquired into 32 K data points over a spectral width of 12 kHz, with 8 dummy scans and 64 scans per sample and an additional longitudinal relaxation recovery delay of 3.5 s per scan, giving a total recycle time of 5 s. Phasing and baseline correction were performed in iNMR (Nucleomatica, Molfetta, Italy). Spectra were imported into Matlab using in-house code; we have previously assigned the majority of the NMR-visible resonances in LB (Behrends et al. 2009). Supernatant (exometabolome) spectra were normalized with respect to the internal standard TSP, i.e. such that absolute concentration differences were compared. Cell extract (endometabolome) spectra were normalized to a constant total spectral area, i.e. such that relative concentration differences were compared. All experiments were performed in triplicate.

Metabolite quantification using NMR Suite

NMR Suite (Chenomx, Edmonton, Canada) provides computer-assisted manual fitting of individual metabolites for 'targeted profiling' (Weljie et al. 2006). We used this package to compare compound levels in the presence and absence of additional salt. Natively, NMR Suite uses non-normalised data and TSP as the quantification standard. However, to account for the difference in peak intensity/area based on cell number and extraction efficiency, the fitted concentrations needed to be normalised to a biological internal standard. Therefore, fitted targeted metabolite concentrations were normalized relative to the fitted concentration of valine, which was chosen as a 'housekeeping metabolite' here because valine concentrations were highly correlated with the total spectral area of all non-osmoresponsive compounds (R^2=0.95).

Results

To assess osmotic stress resistance, we grew five strains of *B. cenocepacia* in rich medium (Luria Broth, LB) and in LB supplemented with 0.5 M NaCl. As this constitutes severe osmotic stress with salt concentrations akin to seawater, all five strains were clearly impaired in growth (Figure 1). There were, however, clear differences between the strains: J415 and K56-2 were less impaired by osmotic stress than C1394, CEP0511 and J2315.

To get an insight into how osmotic stress affects intracellular metabolism, we extracted the cells grown in the presence or absence of salt for 24 h, and measured metabolite profiles by NMR spectroscopy. The spectra were correlated point-wise against the presence/absence of salt (i.e. values of 1 or 0). The resulting correlation coefficients in spectral order were back-projected onto a representative ^{1}H-NMR spectrum, and visualized according to colour scale (Figure 2). The metabolite resonances universally (i.e. across all or most strains) responsive to osmotic stress were identified based on in-house databases and our previous work (Behrends et al. 2009) as alanine, glutamate

and phenylalanine. Glutamate has also been identified as an osmolyte for the Gram-negative CF pathogen *P. aeruginosa* (D'Souza-Ault et al. 1993). Surprisingly, the known bacterial osmo-protectant metabolites glycine-betaine and trehalose were shown not to be a universal response to osmotic stress. However, visualisation of the relative levels of these two compounds indicated clear differences across the strains and it was apparent that the two least impaired strains, J415 and K56-2, had higher levels of trehalose (K56-2) and glycine-betaine (both J415 and K56-2) than the other investigated strains (Figure 3). To elucidate the changes in the metabolite pool that occur in response to osmotic stress, we quantified the levels of these potential osmo-protectants (alanine, phenylalanine, glutamate, trehalose and glycine-betaine) using the software package NMR Suite.

As shown in Figure 4, osmotic tolerance strategies can be divided into three categories for the five investigated strains:

1) The semi-resistant strain K56-2 induced metabolic changes in response to osmotic stress. The levels of all five osmo-responsive compounds were increased, with the biggest resulting relative concentration changes and also highest relative concentrations overall for that strain found for glycine-betaine (4-fold increase) and trehalose (1.7-fold increase). (Data for changes relative to unsupplemented cultures is given for all strains in Table S1, online supporting information.)
2) C1394, CEP0511 and J2315, the three strains for which growth was most impaired by osmotic stress, induced neither trehalose nor glycine-betaine synthesis to any great degree. Glycine-betaine concentrations were increased by only about 1.5 fold relative to non-stressed levels, and trehalose levels decreased by a similar fraction. In contrast, amino acid levels (alanine, glutamate, phenylalanine) were increased to similar relative concentrations, which for phenylalanine in J2315 was equivalent to a 8.5-fold increase.

3) J415, the other semi-resistant strain, did not induce its resistance, but contained constitutively high levels of glycine-betaine even in unsupplemented medium. Trehalose levels were also induced, by 2.4 fold, but the final relative concentration was still small.

It is a crucial to rule out that the observed differences are not purely due to differences in growth. Therefore, cultures of K56-2, CEP0511 and J415 grown under non-stress conditions were sampled at optical densities similar to those of the osmotically stressed cultures after 24 h (OD ~0.6). While differences were found in the endo-metabolome compared to unstressed 24 h cultures, the relative levels of osmo-protectants remained comparable (data not shown). Therefore, the changes seen between high and low salt levels are truly a physiological response to osmotic stress. As the increased levels of amino acids did not seem to confer any sort of tolerance to osmotic stress, they could be part of a generic stress response. Further studies could aim to elucidate whether different stresses also trigger accumulation of glutamate, alanine and phenylalanine.

Fortuitously, all of the detected osmo-responsive compounds are present and detectable by NMR in LB medium, and so we were able to assess whether the intracellular changes were related to increased utilization from the medium. In general the changes were not based on increased compound uptake; in particular, uptake of amino acids from the media actually decreased in high osmolarity conditions (Figure 5). In contrast to the differences in the endometabolome, the increased levels of amino acids in the medium are likely the result of diminished uptake due to poorer growth. However, the strain with constitutively high levels of glycine-betaine, J415, was the most efficient at taking up glycine-betaine from the medium, in both normal and salt-stressed media. The other resistant strain, K56-2, showed slight but significant increases in the amounts of both trehalose and glycine-betaine taken up from LB under osmotic stress (Figure 5).

Discussion

This study clearly demonstrates three different strategies of osmotic tolerance in this relatively small subset of one species of the Bcc. The findings suggest that *recA* subgroup membership is not a factor predicting osmotic stress tolerance. In the investigated sample set J2315 and K56-2 are both members of the III-A lineage, whereas J415, C1394 and CEP0511 are members of III-B (Baldwin et al. 2005). Interestingly, in both *recA* subgroups, different osmotic tolerance strategies can be observed. For IIIA, K56-2 is far less impacted than J2315 due to its induced accumulation of trehalose and glycine-betaine. For IIB, the constitutively high levels of glycine-betaine in J415 and the resulting improved osmotic tolerance are not seen in C1394 and CEP0511. This may seem surprising at first, but it is not uncommon to see differences in physiological or medically relevant parameters within a given *recA* lineage. An example is the difference in mortality rates observed between two outbreaks caused by *recA* lineage IIIA strains in Manchester and Prague, respectively (Drevinek and Mahenthiralingam 2010).

In this context, it is important to note that while interesting from a fundamental physiological point of view, the salt levels in this study (that are akin to sea water) are clearly not a physiological state that can be directly compared to conditions in the CF lung (Smith *et al*., 1996). However, as previous studies indicated that *B. cenocepacia* produces a toxin and induced the production of cable pili under CF-lung-like osmotic stress with the latter being associated with the ET-12 transmissible lineage and with an apparent role in mediating invasion of epithelial cells (Urban, *et al*., 2005; Huber, 2004; Tomich and Mohr, 2004), future studies could investigate osmotic tolerance and strategies at physiological salt conditions.

It is possible that the inducible resistance of K56-2 is related to the putative pleiotropic regulator Pbr (Ramos et al. 2010). The gene was recently identified in a virulence screen

of K56-2 mutants against the nematode *Caenorhabditis elegans*. A *pbr* mutant showed attenuated virulence and various other phenotypes, including reduced osmotic tolerance. Interestingly, *pbr* is unique to K56-2 among sequenced *Burkholderia* strains. As the osmotic tolerance strategy of K56-2 is also unique among the investigated *B. cenocepacia* strains, it is possible the gene plays a role in inducing vital osmotic tolerance pathways. To gain a deeper level understanding of the osmotic stress tolerance in K56-2, further studies could focus on how comparing metabolite levels and fluxes in K56-2 and the *pbr* mutant in both stressed and unstressed conditions.

In summary, the observed differences in osmotic tolerance strategies illustrate the complexity and flexibility of Bcc physiology. Our results failed to disprove the null hypothesis that the strains of *B. cenocepacia* would have no unique metabolic responses to osmotic stress, but instead defined three different strategies that were of varying utility against high salt levels: high levels of osmoprotectant compounds even in normal medium (semi-tolerant); increases in osmoprotectant compounds in response to osmotic stress (semi-tolerant); and increases in amino acid concentrations in response to osmotic stress (non-resistant). While the study is only a first look at stress-related metabolic changes in *B. cenocepacia*, it elucidated interesting differences and generated testable hypotheses.

Acknowledgements

VB was funded by Imperial College London. Work in HDW's laboratory was funded by the BBSRC and The Wellcome Trust. The authors thank Dr Olaf Beckonert for technical assistance with NMR spectroscopy, Dr Ben Ryall for Bcc strain curation and Prof. Jeremy Nicholson for support and access to facilities.

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Figure legends

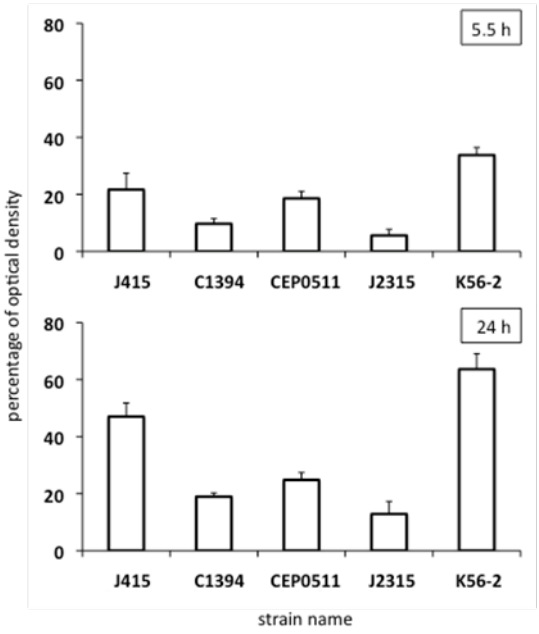
Figure 1: Growth inhibitory effects of high osmolarity on five *B. cenocepacia* strains. Optical density was recorded at the indicated time-points for the strains grown in LB in presence and absence of 0.5 mol l^{-1} NaCl salt in the medium. Growth is expressed as percentage of optical density reached in the unsupplemented cultures. Error bars represent S.D. (n=3).

Figure 2: Global analysis of concentration changes in response to growth in high osmolarity media. A linear correlation analysis was performed using ^{1}H NMR spectra of extracts of *B. cenocepacia* grown in unsupplemented LB and LB supplemented with 0.5 mol l^{-1} NaCl. (a): alanine. (b): glutamate. (c): phenylalanine.

Figure 3: Levels of known osmo-protectants in extracts of *B. cenocepacia* grown in LB supplemented with 0.5 mol l^{-1} NaCl. A: Trehalose, B: Glycine-betaine. Error bars represent S.D. (n=3).

Figure 4: Strain-specific intracellular metabolite changes in response to growth in high osmolarity media. The area of the circle represents relative concentration. Black circles represent concentrations for LB-grown cultures, red circles represent concentrations for cultures grown in LB supplemented with 0.5 mol l^{-1} NaCl.

Figure 5: Strain-specific extracellular metabolite changes in response to growth in high osmolarity media, i.e. a large circle represents a large amount remaining in stationary phase and therefore poor utilization of that substrate. The area of the circle represents absolute concentrations, the area equivalent to 1 mM is given in the top left corner. Black circles represent extracellular concentrations for LB-grown cultures, red circles represent concentrations for cultures grown in LB supplemented with 0.5 mol l^{-1} NaCl.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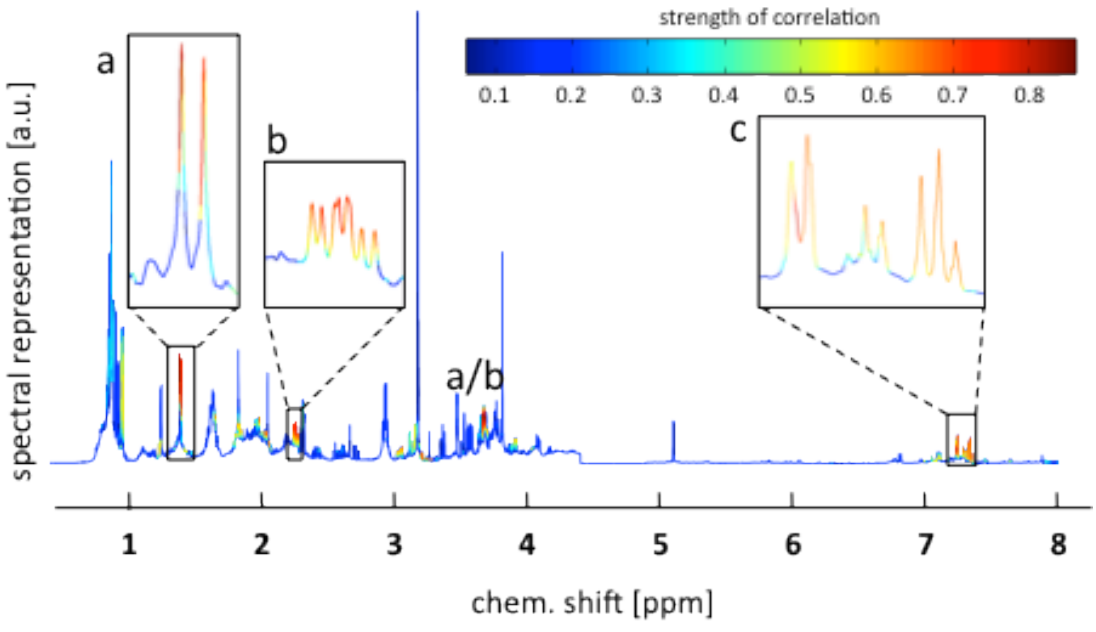
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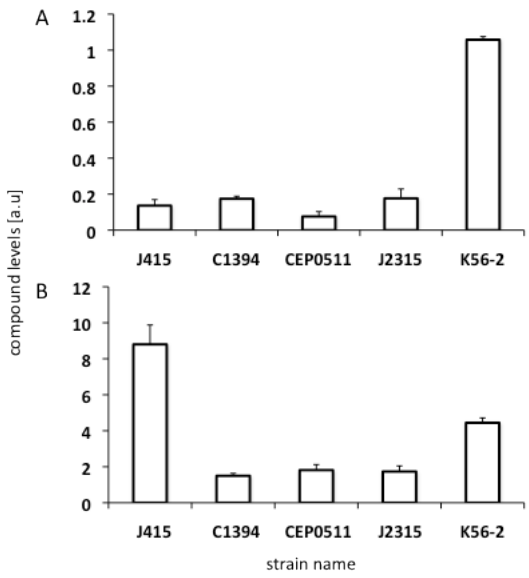
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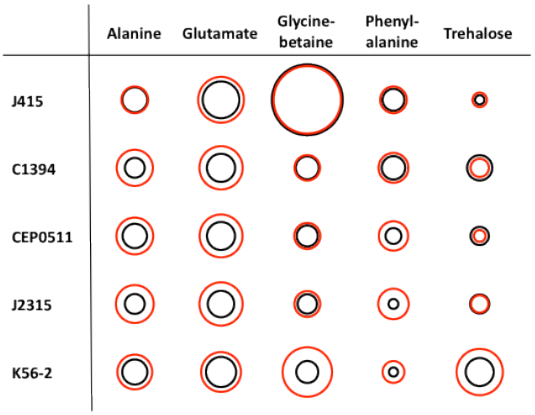
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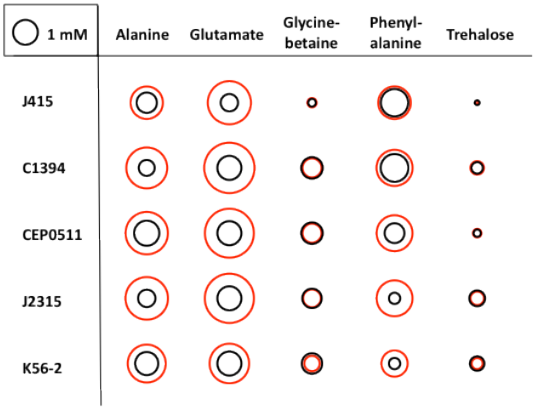
1 mM
Alanine
Glutamate
Glycine-betaine
Phenyl-alanine
Trehalose
J415
C1394
CEP0511
J2315
K56-2

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Supplementary information

Tables legends

Table S1: Changes and S.D. (n=3) in percent of the wild-type of osmotically responsive metabolites in osmotically stressed *B. cenocepacia* cells relative to concentrations found in cells grown in unsupplemented LB, i.e 0 = no change.

Table S2: Changes and S.D. (n=3) in percent of the wild-type of osmotically responsive metabolites in supernatants of osmotically stressed *B. cenocepacia* cultures relative to concentrations found in supernatants of cultures grown in unsupplemented LB, i.e 0 = no change.

Tables

Table S1

	Alanine		**Glutamate**		**Glycine-betaine**		**Phenylalanine**		**Trehalose**	
	AVG	S.D.	AVG	S.D.	AVG	S.D.	AVG	S.D.	AVG	S.D.
J415	13.9	29.59	55.9	24.51	-9.7	10.25	50.5	22.41	143.8	63.42
C1394	228.3	41.26	133.4	24.95	24.2	0.63	65.0	33.24	-48.2	32.91
CEP0511	127.1	12.46	133.1	8.87	51.8	3.07	247.3	11.99	-61.3	52.48
J2315	257.0	18.66	165.6	7.03	78.7	2.90	849.1	11.28	-16.3	101.80
K56-2	91.3	28.11	74.0	14.30	403.1	2.99	475.1	13.67	172.8	26.32

Table S2

	Alanine		Glutamate		Glycine-betaine		Phenylalanine		Trehalose	
	AVG	S.D.	AVG	S.D.	AVG	S.D.	AVG	S.D.	AVG	S.D.
J415	150.3	23.78	499.0	23.32	47.3	42.48	40.8	5.68	-37.4	16.88
C1394	545.1	21.05	338.6	19.77	-21.4	20.63	69.9	2.78	35.4	3.34
CEP0511	182.1	2.96	316.5	32.99	-20.3	13.53	227.5	2.14	27.0	171.74
J2315	492.9	32.22	320.5	18.93	-16.2	16.18	902.9	2.62	-27.6	5.86
K56-2	166.8	3.24	171.2	23.52	-43.7	4.37	429.8	5.48	-42.3	5.12