1 **Differences in strategies to combat osmotic stress in *Burkholderia***

2 ***cenocepacia* elucidated by NMR-based metabolic profiling**

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25 Running title: B. cenocepacia osmotic stress tolerance

1 Abstract

2 **Aims**: To investigate mechanisms of osmotic tolerance in *Burkholderia cenocepacia,* a

3 member of the *B. cepacia* complex (Bcc) of closely related strains, which is of clinical as

4 well as environmental importance.

5 **Methods and Results**: We employed NMR-based metabolic profiling (metabolomics) to

6 elucidate the metabolic consequences of high osmotic stress for five isolates of

7 *B. cenocepacia*. The strains differed significantly in their levels of osmotic stress

8 tolerance, and we identified three different sets of metabolic responses with the strains

9 least impacted by osmotic stress exhibiting higher levels of the osmo-protective

10 metabolites glycine-betaine and / or trehalose. Strains either increased concentrations

11 or had constitutively high levels of these metabolites.

12 **Conclusions**: Even within the small set of *B. cenocecepacia* isolates, there was a

13 surprising degree of variability in the metabolic responses to osmotic stress.

14 **Significance and impact of the study**: The metabolic responses, and hence osmotic

15 stress tolerance, varies between different *B. cenocepacia* isolates. This study provides a

16 first look into the potentially highly diverse physiology of closely related strains of one

17 species of the Bcc and illustrates that physiological or clinically relevant phenotypes are

18 unlikely to be inferable from genetic relatedness within this species group.

19

1 Introduction

2

3 Cystic fibrosis (CF) is a monogenic multisystem disorder that renders patients immuno-

4 compromised and predisposes them to fungal and bacterial lung infections (Davies et al.

5 2007). Opportunistic pathogens like *Pseudomonas aeruginosa* establish long-term

6 infections that can last for decades, lead to a downward spiral of inflammation and loss

7 of lung function and are the major cause of morbidity and mortality today (Davies 2002;

8 Davis 2006). In addition to *P. aeruginosa*, infections with methicillin-resistant

9 *Staphylococcus aureus* or a member of the *Burkholderia cepacia* complex (Bcc) are also a

10 great cause of concern. The latter can lead to cepacia syndrome, a rapid deterioration of

11 lung function associated with septicaemia and early patient death (Mahenthiralingam et

12 al. 2002; Davis 2006).

13

14 The Bcc is a closely related species group with at least 17 species (Coenye and

15 Vandamme 2003; Vanlaere et al. 2008; Vanlaere et al. 2009). The taxonomy is complex

16 and ecological diversity is high, with ecological significance ranging from plant growth

17 promoting agent to known plant pathogen (Mahenthiralingam et al. 2005). Two species

18 of the Bcc, *B. cenocepacia* and *B. multivorans,* account for more than 85 % of all Bcc

19 isolates recovered from the CF lung. While *B. multivorans* is has recently supplanted

20 *B. cenocepacia* as the most commonly isolated Bcc species in most of the world,

21 *B. cenocepacia* is still of high clinical importance (Govan et al. 2007; LiPuma 2010).

22

23 The close genetic proximity of Bcc isolates renders traditional means of species

24 assignment problematic and approaches based on 'housekeeping genes' were developed

25 to provide a quick way to identify Bcc isolates to strain level. Multi-locus sequence

26 typing (MLST) of seven house-keeping genes has become an accepted technique to

1   identify Bcc members (Baldwin et al. 2005). For *B. cenocepacia*, the species is divided

2   into subgroups based on the sequence of the DNA recombinase gene *recA* (III-A to III-E)

3   (Mahenthiralingam et al. 2000; Baldwin et al. 2005). There are several epidemic lineages

4   within *B. cenocepacia*, most prominently the ET-12 lineage, which falls into the *recA* III-A

5   subgroup (Mahenthiralingam et al. 2005). Strains of these lineages are highly

6   transmissible and cause patient-to-patient spread (Mahenthiralingam et al. 2005;

7   Drevinek and Mahenthiralingam 2010). While it is not completely understood which

8   factors enable ET-12 strains to cause infections in the CF lung, the genome of ET-12

9   strain J2315 contains an unusually high fraction of pathogenicity islands (Holden et al.

10   2009; Drevinek and Mahenthiralingam 2010).

11   Outside the CF lung, *B. cenocepacia* strains have been isolated from the rhizosphere of

12   wheat and maize (Coenye and Vandamme 2003), where they have been shown to act as

13   protection against fungal infection and plant growth promoting agents (Bevivino et al.

14   2005).

15

16   Survival in both environments depends on maintenance of a stable population size and

17   resistance to or at least some sort of tolerance of abiotic and biotic stress. Osmotic stress

18   could potentially be a factor in both the lungs of CF patients (Smith et al., 1996) as well

19   as the rhizosphere. We investigated the osmotic stress tolerance of five *B. cenocepacia*

20   isolates and elucidated the metabolic changes associated with osmotic stress using

21   nuclear magnetic resonance (NMR)-based metabolic profiling. NMR spectroscopy has

22   the advantage of near universal detection, but suffers from relatively low detection

23   thresholds (Grivet et al. 2003). Because osmo-protectants have to be present in the cell

24   in relatively high concentrations and are not restricted to one particular compound

25   class, NMR spectroscopy is ideally suited for measuring metabolic changes in response

26   to high osmolarity and has been successfully used previously to characterise osmotic

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

2   2010; Gavaghan et al. 2010).

3

4   We found that within the five investigated *B. cenocepacia* and even within the two ET-12

5   strains, several metabolic strategies exist to counteract osmotic stress. At high salt

6   levels, strains that accumulated the classic osmo-protectants trehalose and glycine-

7   betaine were less impacted by osmotic stress than strains that increased the

8   intracellular concentration of certain amino acids.

9   **Methods**

10

11   *Bacterial strains*

12   The following *B. cenocepacia* strains were obtained from the BCCM library (Ghent,

13   Belgium) and maintained as glycerol stocks at -80 °C: III-A lineage (and ET-12 lineage):

14   J2315 (LMG 16656/ST28), K56-2 (LMG 18863/ST30), III-B lineage: J415 (LMG 16654;

15   ST34), C1394 (LMG 16659; ST35), and CEP0511 (LMG 18830; ST39); sequence types

16   (ST) were taken from Baldwin *et al*. (2005)**.** For pre-cultures, the bacteria were

17   inoculated in lysogeny broth (LB) (tryptone, 10 g l^{-1}, yeast extract, 5 g l^{-1}, and NaCl, 5 g l^{-1}

18   ^{1}) from fresh plates and grown for 16 h. The strains were inoculated to identical starting

19   optical densities from pre-culture into 25 ml universal tubes either containing 10 ml LB

20   supplemented with 0.5 mol l^{-1} NaCl (29.2 g l^{-1}) or 10 ml LB supplemented with an equal

21   volume of water (unsupplemented LB). Cultures were grown at 37 °C for 24 h at 200

22   rpm orbital shaking.

23

24   *Metabolite analysis*

25   To measure the strains' media utilisation, 1 ml of the culture was sampled, the optical

26   density was recorded and 750 µl of cell-free supernatant were mixed with 200 µl ^2H_2O

1   containing 5 mmol l^{-1} trimethylsilyl-1-propionic acid-d_4 (TSP), 25 mol l^{-1} sodium azide

2   and 50 µl of 1 mol l^{-1} phosphate buffer, pH 7. The ^2H_2O provided a field frequency lock

3   for the spectrometer and the TSP served as an internal chemical shift reference. To

4   quantify the endo-metabolome (i.e. the intracellular metabolites), the sample was

5   centrifuged for 5 min (RT, 3600 x g), the pellet washed with 5 ml quarter strength

6   Ringer's solution and centrifuged again. Pellets were resuspended in 5 ml MeOH-water

7   75:25 % (v/v, -20 °C), sonicated for 10 min and vacuum dried. Finally, the pellets were

8   resuspended in 0.6 ml 0.1 mol l^{-1} phosphate, pH 7.0, 90 % D_2O with 1 mmol l^{-1} TSP.

9   Spectra were acquired on a Bruker Avance DRX600 NMR spectrometer (Bruker BioSpin,

10   Rheinstetten, Germany), with a magnetic field strength of 14.1 T and resulting ^1H

11   resonance frequency of 600 MHz, equipped with a 5 mm inverse flow probe. Samples

12   were introduced using a Gilson flow-injection autosampler. Spectra were acquired

13   following the approach given in (Beckonert et al. 2007). Briefly, a one-dimensional

14   NOESY pulse sequence was used for water suppression; data were acquired into 32 K

15   data points over a spectral width of 12 kHz, with 8 dummy scans and 64 scans per

16   sample and an additional longitudinal relaxation recovery delay of 3.5 s per scan, giving

17   a total recycle time of 5 s. Phasing and baseline correction were performed in iNMR

18   (Nucleomatica, Molfetta, Italy). Spectra were imported into Matlab using in-house code;

19   we have previously assigned the majority of the NMR-visible resonances in LB

20   (Behrends et al. 2009). Supernatant (exometabolome) spectra were normalized with

21   respect to the internal standard TSP, i.e. such that absolute concentration differences

22   were compared. Cell extract (endometabolome) spectra were normalized to a constant

23   total spectral area, i.e. such that relative concentration differences were compared. All

24   experiments were performed in triplicate.

25

26   *Metabolite quantification using NMR Suite*

1 NMR Suite (Chenomx, Edmonton, Canada) provides computer-assisted manual fitting of

2 individual metabolites for 'targeted profiling' (Weljie et al. 2006). We used this package

3 to compare compound levels in the presence and absence of additional salt. Natively,

4 NMR Suite uses non-normalised data and TSP as the quantification standard. However,

5 to account for the difference in peak intensity/area based on cell number and extraction

6 efficiency, the fitted concentrations needed to be normalised to a biological internal

7 standard. Therefore, fitted targeted metabolite concentrations were normalized relative

8 to the fitted concentration of valine, which was chosen as a 'housekeeping metabolite'

9 here because valine concentrations were highly correlated with the total spectral area of

10 all non-osmoresponsive compounds (R^2=0.95).

Results

12 To assess osmotic stress resistance, we grew five strains of *B. cenocepacia* in rich

13 medium (Luria Broth, LB) and in LB supplemented with 0.5 M NaCl. As this constitutes

14 severe osmotic stress with salt concentrations akin to seawater, all five strains were

15 clearly impaired in growth (Figure 1). There were, however, clear differences between

16 the strains: J415 and K56-2 were less impaired by osmotic stress than C1394, CEP0511

17 and J2315.

18

19 To get an insight into how osmotic stress affects intracellular metabolism, we extracted

20 the cells grown in the presence or absence of salt for 24 h, and measured metabolite

21 profiles by NMR spectroscopy. The spectra were correlated point-wise against the

22 presence/absence of salt (i.e. values of 1 or 0). The resulting correlation coefficients in

23 spectral order were back-projected onto a representative ^{1}H-NMR spectrum, and

24 visualized according to colour scale (Figure 2). The metabolite resonances universally

25 (i.e. across all or most strains) responsive to osmotic stress were identified based on in-

26 house databases and our previous work (Behrends et al. 2009) as alanine, glutamate

1   and phenylalanine. Glutamate has also been identified as an osmolyte for the Gram-

2   negative CF pathogen *P. aeruginosa* (D'Souza-Ault et al. 1993). Surprisingly, the known

3   bacterial osmo-protectant metabolites glycine-betaine and trehalose were shown not to

4   be a universal response to osmotic stress. However, visualisation of the relative levels of

5   these two compounds indicated clear differences across the strains and it was apparent

6   that the two least impaired strains, J415 and K56-2, had higher levels of trehalose (K56-

7   2) and glycine-betaine (both J415 and K56-2) than the other investigated strains

8   (Figure 3). To elucidate the changes in the metabolite pool that occur in response to

9   osmotic stress, we quantified the levels of these potential osmo-protectants (alanine,

10   phenylalanine, glutamate, trehalose and glycine-betaine) using the software package

11   NMR Suite.

12   As shown in Figure 4, osmotic tolerance strategies can be divided into three categories

13   for the five investigated strains:

14   1) The semi-resistant strain K56-2 induced metabolic changes in response to

15   osmotic stress. The levels of all five osmo-responsive compounds were

16   increased, with the biggest resulting relative concentration changes and also

17   highest relative concentrations overall for that strain found for glycine-betaine

18   (4-fold increase) and trehalose (1.7-fold increase). (Data for changes relative to

19   unsupplemented cultures is given for all strains in Table S1, online supporting

20   information.)

21   2) C1394, CEP0511 and J2315, the three strains for which growth was most

22   impaired by osmotic stress, induced neither trehalose nor glycine-betaine

23   synthesis to any great degree. Glycine-betaine concentrations were increased by

24   only about 1.5 fold relative to non-stressed levels, and trehalose levels decreased

25   by a similar fraction. In contrast, amino acid levels (alanine, glutamate,

26   phenylalanine) were increased to similar relative concentrations, which for

27   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

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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.
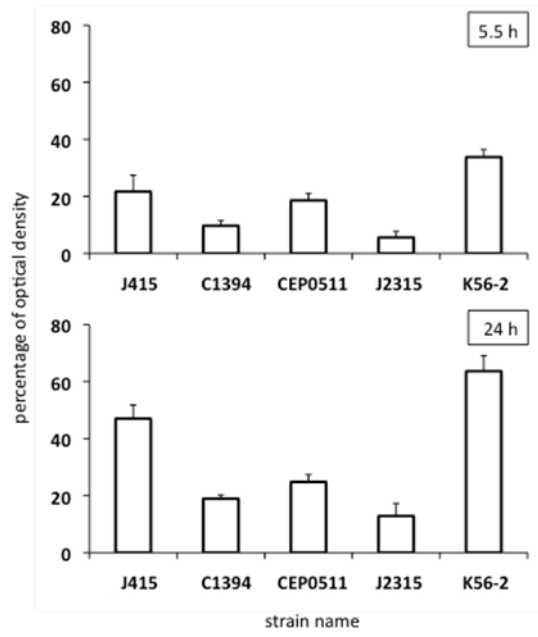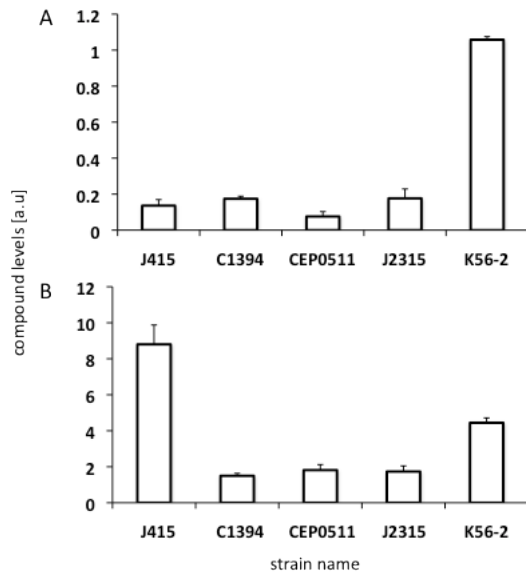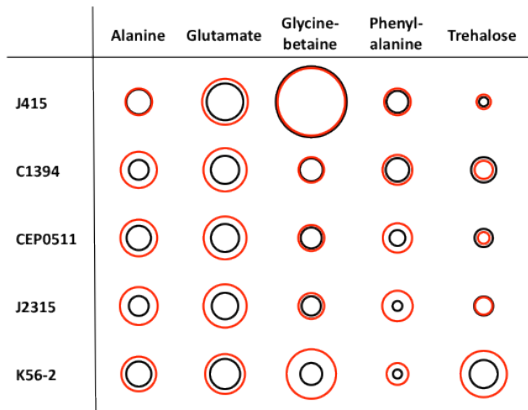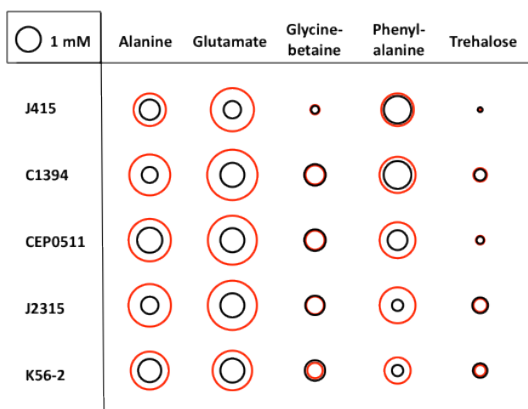
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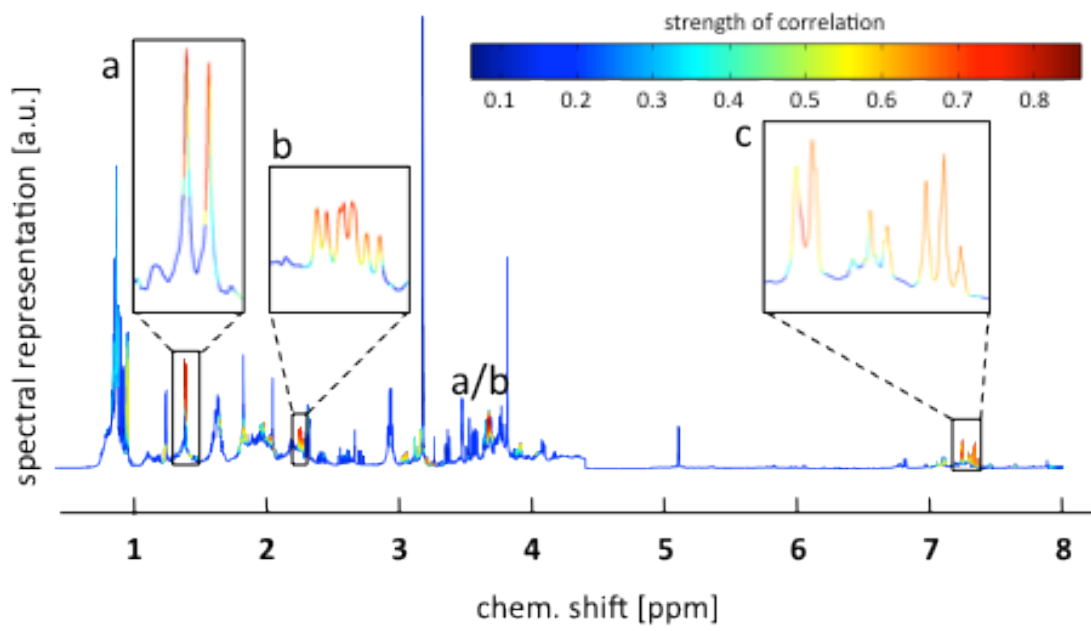
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1 Supplementary information

2 Tables legends

3 Table S1: Changes and S.D. (n=3) in percent of the wild-type of osmotically responsive

4 metabolites in osmotically stressed *B. cenocepacia* cells relative to concentrations found

5 in cells grown in unsupplemented LB, i.e 0 = no change.

6 Table S2: Changes and S.D. (n=3) in percent of the wild-type of osmotically responsive

7 metabolites in supernatants of osmotically stressed *B. cenocepacia* cultures relative to

8 concentrations found in supernatants of cultures grown in unsupplemented LB, i.e 0 =

9 no change.

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12 Tables

13 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

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1   **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

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