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Metabolite profiling of the cold adaptation of *Pseudomonas putida* KT2440 and cold-sensitive mutants

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Abstract

Free-living bacteria such as *Pseudomonas putida* are frequently exposed to temperature shifts and non-optimal growth conditions. We compared the transcriptome and metabolome of the cold adaptation of *Pseudomonas putida* KT2440 and isogenic cold-sensitive transposon mutants carrying transposons in their *cbrA, cbrB, pcnB, vacB* and *bipA* genes. *P. putida* changes the mRNA expression of about 43% of all annotated ORFs during this initial phase of cold adaptation, but only a small number of six to 93 genes were differentially expressed at 10°C between wild type strain and the individual mutants. The spectrum of metabolites underwent major changes during cold adaptation particularly in the mutants. Both KT2440 strain and the mutants increased the levels of the most abundant sugars and amino acids which were more pronounced in the cold-sensitive mutants. All mutants depleted their pools for core metabolites of aromatic and sugar metabolism, but increased their pool of polar amino acids which should be advantageous to cope with the cold stress.

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Introduction

Its metabolic versatility, degradative potential, and ability to colonize bulk soil and the rhizosphere make *Pseudomonas putida* an ideal candidate for genetic engineering and applications in biotechnology, bioremediation, and agriculture (Wu et al., 2011). Strain KT2440 is one of the best characterized pseudomonads (Nelson et al., 2002; Regenhardt et al., 2002) and has been optimized as a 'laboratory workhorse' for biotechnology (Dvořák and de Lorenzo, 2018; Martínez-García and de Lorenzo, 2019) and systems biology (Sudarsan et al., 2014), but it has retained its ability to survive and function in the environment.

Free-living bacteria are frequently exposed to temperature shifts and non-optimal growth conditions. In order to grow at low temperatures, a microorganism must overcome the growth-limiting effects of this stress condition. We previously had screened a transposon library for genes that are essential for the survival of *P. putida* KT2440 at low temperatures (Reva et al., 2006). The CbrAB two component system controlling catabolite repression and co-ordinating carbon metabolism (Valentini et al., 2014; Barroso et al., 2018), PcnB and VacB, which control mRNA stability (Hester et al., 2000), and BipA, which exerts transcript-specific translational control (Yuste et al., 2006), were essential to cope with cold stress. Here we report on the comparison of the transcriptome and metabolome of the cold adaptation of wild-type KT2440 and isogenic cold-sensitive transposon mutants carrying transposons in their *cbrA, cbrB, pcn, vacb* and *bipA* genes, respectively.

Results and Discussion

Growth characteristics during cold stress

Screening of the *P. putida* KT2440 plasposon library had revealed that mutants carrying the plasposon in eight genes, namely *cysM, nuoL, PP4646, cbrA, cbrB, pcnB, vacB* and *bipA* (Suppl. Table1

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– strain characteristics) were not growing at 4 °C when cultured in microtiter plates in M9 medium with 15 mM benzoate as sole carbon source (Reva et al., 2006). When we re-tested these mutants in cultures of 20 mL under shaking at 4 °C, the mutants in PP4646, *cysM* or *nuoL* were only slightly compromised in growth. Hence these three targets were not examined further. On the other hand, consistent with the initial screen no growth was detectable with the *cbrA, cbrB, pcnB, vacB* and *bipA* mutants.

These five genes encode central functions in the *P. putida* cell and our experiments added the information that they are essential to cope with cold stress. BipA is a master regulator of translation (Yuste et al., 2006), the 3',5' exoribonuclease VacB cleaves poly(A), poly(U) and rRNAs (Hester et al., 2000) and the operon *cbrA – cbrB – crcZ – pcnB* controls the utilization of carbon sources and modulates mRNA stability by polyadenylation (Amador et al., 2016; Barroso et al., 2018; Fonseca et al., 2013; García-Mauriño et al., 2013; Hernández-Arranz et al., 2016; La Rosa et al., 2015; Moreno et al., 2012; Sánchez-Hevia et al., 2018; Tsipa et al., Valentini et al., 2014) . The central role of these genes for *P. putida* suggests that their inactivation affects numerous features of lifestyle and metabolism. Hence we next searched for the temperature that still keeps these mutants growing but is most informative to resolve the adaptation of these mutants to lower temperatures.

Prior to testing different temperatures, we switched from benzoate as sole carbon source to succinate which had been chosen as the reference compound in studies on the systems biology of *P. putida* (Nogales et al., 2008; Park et al., 2009; Daniels et al., 2010; Nikel et al., 2014; La Rosa et al., 2015; Hintermayer and Weuster-Botz, 2017; Tsipa et al., 2017). Wild type and mutants were cultured in M9 medium with 3, 10, 15 or 20 mM succinate. No or poor growth were observed with 3 and 10 mM succinate, whereas the typical behavior of lag phase, exponential growth and smooth transition to stationary phase was seen with both 15 mM and 20 mM succinate. Next, the cells were cultured

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with succinate as carbon source at 5, 10, 23 and 30 °C. Growth rates were indistinguishable at 23°C and 30°C. Minimal or no growth was seen for the mutants at 5°C and growth was retarded at 10°C. Thus the experimental conditions for cold adaptation were set to growth with 15 mM succinate at 10°C.

Transcriptome and metabolome of cold adaptation

The KT2440 wild type strains and the five plasposon mutants were grown in a 1.5 L batch cultures using the BioFlo 110 fermenter at 30°C from OD_{600} 0.05 until 0.8. After taking samples, the fermenter was cooled within 45 minutes to 10°C and then maintained at 10°C for 2 hours until final sampling. We previously reported that *P. putida* changes the mRNA expression of about 43% of all annotated ORFs during this initial phase of cold adaptation (Frank et al., 2011). In contrast to this vast number of changes in the global transcriptome only a comparably small number of six to 93 genes were differentially expressed at 10°C between wild type strain and the individual mutants (Suppl. Table S2). Interestingly, all five mutants showed a consistent down-regulation of *ped* genes (operon PP2663 – PP2682) involved in the degradation of 2-phenylethanol and aliphatic alcohols (C5 – C10) (Arias et al., 2008). The PedS2/PedR2 two-component system (PP2671/2672) within the operon that is crucial for the rare earth element switch in *P. putida* KT2440 (Wehrmann et al., 2018) was not differentially regulated.

In contrast to the comparably small changes in the transcriptome the spectrum of metabolites underwent major changes during cold adaptation particularly in the mutants (see Supplementary information 1 for experimental detail). Principal component analysis (Figure 1, Suppl. Table S3) revealed that the wild type strain maintained its metabolic profile, whereas peculiarly the *cbrA* and *pcnB* transposon mutants showed strong individual changes in their metabolic profiles. Even more remarkably, wild type strain and each mutant exhibited a strain specific signature of its metabolome

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at both temperatures (Figure 2, Suppl Figure 1 and Table S4). In other words, the metabolic profiles of the strain at two temperatures were more similar to each other than the metabolomes of the strain panel at either 30°C or 10°C.

Table 1 lists the concentrations of the 20 metabolites most abundant in KT2440 wild type and mutant strains at 10°C and 30°C. During cold adaptation KT2440 and the mutants consistently increased the levels of lactate and of the sugars fructose-6-phosphate, glucose-6-phosphate and of the amino acids valine and glutamic acid the latter also the major driver of intermediary metabolism in pseudomonads (Frimmersdorf et al., 2010). *P. putida* accumulated its most prevalent sugar and amino acids to cope with the metabolic demands at lower temperature. The mutants behaved like wild type implying that this adaptation of the most abundant metabolites did not require the key sensors of cold stress.

Principal component analysis segregated wt and mutants primarily by strain and not by temperature indicating that mutant-associated shifts should already be partly visible at the indifferent temperature of 30°C. Compared to the wild type KT2440 strain, all cold-stress sensitive mutants had depleted pools for core metabolites of aromatic (benzoate) and sugar metabolism (pyruvate, glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate) and the direct conversion products of the most abundant metabolite glutamic acid, i.e. oxoproline and oxoglutarate. Moreover pools were low for peripheral mono- or disaccharides such as galactose, xylulose-5-phosphate and N-acetylglucosamine. Instead, the mutants kept higher levels of the membrane-disorganizing fatty acid dodecanoic acid utilized for lipid A biosynthesis and increased the pool of amino acids, i.e. homoserine, tartaric acid, isoleucine and proline. After cold adaptation to 10°C wild type and mutant had increased the levels of glutamate and lactate, but in addition the cold-stress sensitive mutants had accumulated glutamine, aspartate and the hydroxyproline derivative 1-pyrroline-3-hydroxy-5-

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carboxylate (Koo and Adams, 1974) in their cells as highly abundant compounds (Table 1). With the exception of the *cbrB*::Tn*5*, the pool of glutamate was more than twofold larger in the mutants than in wild type indicating that the mutants had to keep higher levels to cope with the challenges of cold stress

The strain *P. putida* S12 had been cultured under similar conditions at 30°C in a fermenter with succinate as sole carbon source, however, the substrate concentration of 165.4 mM was 11-fold higher (van der Werf et al., 2008) than in our experiments. The concentrations of intermediates of the central carbon metabolism, namely pyruvate, glucose, glucose-6-phosphate and fructose-6-phosphate were in the same range, but AMP, mannitol and trehalose were several orders of magnitude more abundant in the S12 strain (Suppl. Table S5). We hypothesize that the larger supply of succinate enabled the S12 cell to store the osmolytes mannitol and trehalose conferring tolerance to desiccation and organic solvents.

In summary, the disruption of key genes of the adaptation to cold stress led to mutant-specific metabolic changes consistently observed at both 30°C and 10°C (Suppl. Figure S1). During cold adaptation to 10°C similar shifts were seen in wild type strains and isogenic mutants for numerous metabolites, but in contrast to wild type the cold-sensitive mutants accumulated higher levels of polar amino acids including 1-pyrroline-3-hydroxy-5-carboxylate, aspartate, glutamine and glutamate, one of the key compounds in pseudomonads. Thus a larger pool of polar amino acid metabolites emerged when the core genetic elements of adaptation to cold stress had been inactivated. However, in addition to this trend consistently observed in all mutants, each mutant exhibited an individual metabolome profile. This finding was somewhat unexpected for the *cbrAB* two-component system because the histidine kinase CbrA and its response regulator CbrB synergize in carbon metabolism and the uptake of amino acids (Li and Lu, 2007; Monteagudo-Cascales E et al.,

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2019). However, CbrB also indirectly regulates the 'Carbon Repression Control' (García-Mauriño SM et al., 2013; Barroso et al., 2018). In other words, features and targets not shared by CbrA and CbrB and the transposon-mediated loss of CbrAB regulation by phosphorylation may explain the different composition of the mutant CbrA and CbrB metabolomes.

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Figure Legend

Figure 1. Principal component analysis of the profiles of 147 metabolites of *P. putida* KT2440 and isogenic transposon mutants grown in M9 mineral medium supplemented with 15 mM succinate at 10°C and 30°C. The evaluation was based on normalized logarithmically transformed mean peak areas of two biological replicates independently processed at least in triplicate (see Supplementary Information for experimental details).

Figure 2. Heatmap presentation of the metabolic profiles of *P. putida* KT2440 and isogenic transposon mutants. The figure depicts hierarchical clustering of the normalized logarithmically transformed mean peak areas of the 147 commonly detected metabolites.

Table 1. Concentrations [µg/g dry weight] of the 20 most abundant metabolites in KT2440 wild type and mutant strains at 10°C and 30°C. Data are normalized to 100% ribitol as internal standard.

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Metabolite	KT2440 wt		*cbrA*::Tn5		*cbrB*::Tn5		*pcnB*::Tn5		*vacB*::Tn5		*bipA*::Tn5	
	30°C	10°C	30°C	10°C	30°C	10°C	30°C	10°C	30°C	10°C	30°C	10°C
glutamic acid	105.5	151.9	108.0	346.8	93.6	160.7	126.0	389.0	165.2	347.8	155.7	347.2
ribitol	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
putrescine	12.1	7.5	9.5	14.4	10.7	9.4	21.7	26.6	31.9	30.5	18.1	22.7
palmitic acid	11.3	7.8	17.2	17.2	11.3	16.8	34.2	30.5	37.4	35.7	43.8	51.3
pyruvic acid	11.1	10.8	0.3	1.5	0.5	0.7	1.0	0.0	0.0	0.0	0.3	0.0
glucose 6-phosphate	9.8	33.3	5.6	70.7	1.1	7.8	2.6	57.9	8.3	97.9	7.3	94.5
lactic acid	9.5	17.3	10.2	19.1	3.2	7.0	7.9	20.0	3.1	12.5	2.4	16.8
valine	9.1	73.1	13.5	100.4	6.4	4.7	31.4	241.6	47.9	186.7	26.4	190.7
beta-alanine	8.6	2.1	9.0	3.8	5.6	1.7	6.9	5.5	11.2	7.6	9.0	5.0
aspartic acid	7.5	10.9	7.1	31.8	3.6	11.9	15.9	51.4	19.7	24.9	16.9	65.0
benzoic acid	5.5	1.1	3.0	0.7	0.1	0.3	0.7	1.3	1.5	1.8	2.0	0.9
alanine	5.4	13.2	17.2	29.3	10.8	8.1	25.5	69.6	23.0	60.0	18.0	66.3
1-pyrroline-3-hydroxy-5-carboxylate	5.1	6.9	3.7	16.1	3.1	4.6	9.7	33.1	7.8	17.7	9.0	24.9
oxalic acid	4.4	3.2	6.7	8.0	3.0	6.1	4.4	4.6	20.5	19.9	10.3	13.1
fructose 6-phosphate	4.2	21.7	1.5	26.3	0.2	2.1	0.5	12.9	2.4	45.2	2.1	47.6
glycine	4.0	3.7	1.9	4.2	1.1	3.1	44.0	7.3	6.2	9.4	4.6	10.9
phosphoric acid	3.7	5.2	55.6	93.5	26.8	51.9	135.9	82.5	83.0	100.9	103.2	84.6
N-acetylglutamic acid	3.7	4.4	1.2	7.1	0.3	1.9	4.6	8.8	8.6	5.3	4.1	10.0
threonine	3.0	4.1	3.4	7.4	2.6	2.1	4.7	14.8	6.2	13.0	7.3	16.1
glycerol 3-phosphate	2.9	3.2	1.5	3.0	0.8	1.5	3.0	5.2	2.5	4.5	1.7	4.5
lysine	2.8	1.5	3.4	3.0	2.0	1.7	7.1	11.4	6.7	15.5	6.3	11.3
succinic acid	2.5	3.7	1.3	2.8	0.5	1.0	2.2	4.9	3.1	4.3	2.0	4.9
glutamine	2.5	3.3	1.3	10.5	0.5	1.9	2.4	18.2	4.1	18.7	1.4	20.3
mannose 6-phosphate	2.4	6.2	1.0	11.8	0.1	1.1	0.2	4.7	1.5	21.1	1.3	15.3

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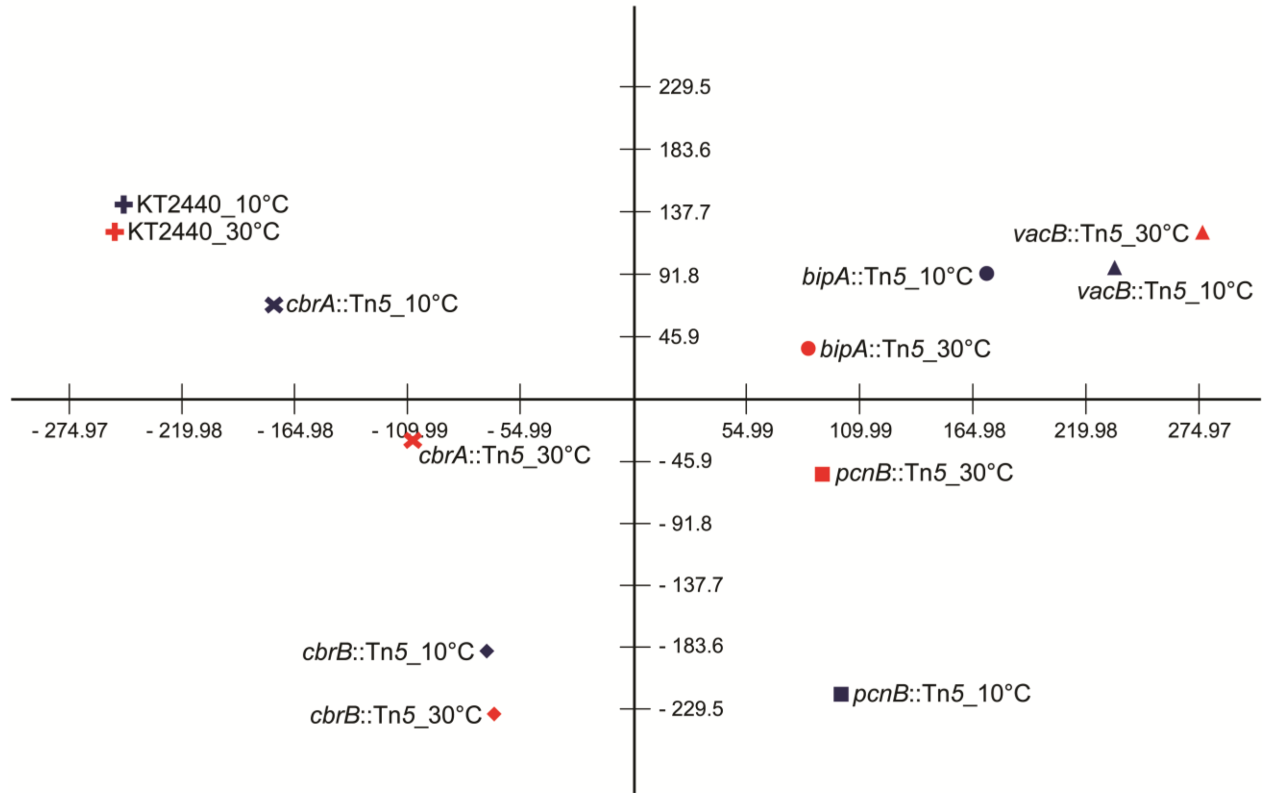

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