Direct assessment of metabolite utilization by *Pseudomonas aeruginosa* from artificial sputum medium

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running title: Metabolite utilization by *Pseudomonas aeruginosa*

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ABSTRACT

We grew *Pseudomonas aeruginosa* in LB and artificial sputum medium (filtered and unfiltered), and quantified metabolite utilization and excretion by NMR spectroscopy (metabolic footprinting, or extracellular metabolomics). Utilization was similar between media, but there were differences in excretion – e.g. acetate was produced only in unfiltered ASM.

MAIN TEXT

The opportunistic pathogen *Pseudomonas aeruginosa* is the major cause of morbidity and mortality for cystic fibrosis (CF) patients (6). In order to better understand *P. aeruginosa* infection it is important to create model conditions that aim to mimic those in the CF lung, such as media designed to mimic the nutritional environment of sputum from CF patients. These include artificial sputum medium (ASM)(11), a complex medium made up using porcine mucin, DNA and amino acids, and synthetic cystic fibrosis medium (SCFM)(9), a defined medium containing mainly amino acids and lactate. In addition, many studies use more conventional rich media like Lysogeny Broth (LB)(4), which is based on tryptone and yeast extract. All of these media support growth of *P. aeruginosa* to high cell numbers, but the question remains how the differences in composition alter the physiology and metabolism of the inoculated bacteria, and hence the relevance to CF infections. Generally, approaches to validating this have depended on indirect means to assess metabolism – for instance, profiling gene transcription (9). However, transcriptional data are often insufficient to capture the actual metabolic changes that occur as a result of perturbing a bacterial cell. Because of this, we wanted to examine the actual changes in metabolite utilization by *P. aeruginosa*, in a comparison of three different complex media: LB, ASM (oASM, prepared according to its original recipe (11)) and a filtered version of ASM (fASM,

prepared according to Kirchner *et al*. (2012) (7)). (We have previously investigated metabolite utilization in SCFM in depth (2), so did not include this medium again here.)

We grew *P. aeruginosa* wild-type PA14 in aerobic conditions in batch culture for 24h (growth conditions described in detail in (2)) and sampled 0.6 ml of culture at nine time points (before inoculation; hourly from two to eight hours; and then at 24h). Of these samples, 0.1 ml was used for measuring optical density for LB and fASM samples (oASM is turbid and OD cannot be used to monitor cell growth). The supernatants were then analysed by NMR spectroscopy as detailed in Behrends *et al*. (2012) (3). The resulting spectra were assigned and integrated for each medium. In total, we were able to assign 19 compounds detectable across all media, of which 18 could be quantified (Table 1; histidine was detected but not integrated here because of pH-induced resonance frequency shifts between spectra) and normalized to compound levels in the uninoculated media. Four further metabolites could be identified in at least two media. Finally, the metabolite concentrations were fitted using non-linear (sigmoid) models, which allows between-media comparisons of uptake and excretion time for individual compounds (2).

Metabolite uptake is tightly controlled and broadly comparable in rich media – There are several possible ways in which media composition could alter bacterial physiology and therefore the bacterial interaction with media. Changes can be qualitative, i.e. the same strain utilizes/excretes a given compound from/into one medium, but not another; or quantitative, i.e. the media composition affects the dynamics of utilization/excretion. By non-linear fitting, the time dimension of a data set is compressed to produce biologically meaningful sigmoid parameters that can detect both qualitative and quantitative changes – e.g. the t_{50} value corresponds to the time at which half of the compound has been taken up (a compound's half-life). Some metabolites, though, had complex utilization profiles, and so standard sigmoid models could not be fitted; e.g. S-oxo-methionine (Figure 1). In total, 14 metabolites were successfully fitted (Table 1). For these compounds, there were no differences between the media after 24h of

growth, as all detectable amino acids (with the exception of methionine, which was not taken up from any medium) were completely depleted after 24h. In addition, the dynamics of uptake were similar across all media at first glance, as the order in which metabolites were taken up was broadly comparable; the most 'outlying' medium, unsurprisingly, was LB: both of the ASM media were more similar to each other than to LB (Figure 2). The two ASM media had essentially identical order of metabolite uptake, but metabolites were taken up slightly more quickly from fASM than from oASM, possibly because oASM also contains alternative carbon sources (macromolecules, lipid droplets). In addition, we also profiled *P. aeruginosa* PAO1 growth on oASM, in order to permit comparison with earlier studies. While there certainly were some differences from PA14, e.g. valine was used earlier by PAO1, the overall pattern of metabolite usage was similar between the two backgrounds (Figure 2).

In agreement with previous studies (2, 8) there was a set of compounds – asparagine, aspartate, alanine, glutamate (and in the case of oASM and fASM also glutamine) – that were taken up early on in growth (Table 1). For these 'early-uptake' compounds, the t_{50} values increased in the order LB to fASM to oASM, i.e. quickest uptake from LB and slowest from oASM. Surprisingly, this order of uptake was reversed for the late-uptake metabolites. Threonine, phenylalanine and tryptophan had t_{50} values of less than12h for all media . In contrast, glycine, lysine, isoleucine and valine were only taken up after 12h from LB, but between 7 to 11h from the ASM media (Table 1; Figure 2). Compound uptake in *Pseudomonas* is tightly controlled by catabolite repression, which affects amino acids and other organic acids as well as carbohydrates (5, 8, 10). The most likely explanation for the surprising delay in the utilization of late-uptake metabolites from LB is catabolite repression by a metabolite found in LB but not in the two ASM media. Trehalose is a good candidate for this metabolite: it is present in LB but not ASM, and was taken up after the early-uptake but before the late-uptake metabolites (Figure 2). There is little information on catabolite repression by trehalose for *Pseudomonas*

87 species (10). In order to test this, we repeated the experiment, but with trehalose spiked into
88 oASM at 2 mM concentration. This demonstrated that trehalose is not, in fact, causing the
89 catabolite repression: the order of metabolite uptake was essentially identical for the two ASM
90 media, regardless of the presence of trehalose (data not shown). The putative repressing
91 compound in LB remains to be elucidated.

92 **Metabolite excretion differs between media**

93 In contrast to uptake, compound excretion was noticeably different between the media. The
94 biggest difference was found when comparing oASM and fASM: acetate was only found in
95 significant amounts in the oASM samples (Figure 1). As the media only differ in a final filtration
96 step, the filtration must remove one or several acetogenic substrates. The turbidity of oASM
97 medium is (at least partly) because of suspended lipid droplets. The lipid fatty acids are
98 metabolized via acetyl-CoA, and so the acetate seen in oASM is potentially the product of
99 overflow metabolism to protect the CoA pool of the cell (12). To test this, we repeated the
100 experiment with oASM, but omitted the egg yolk (a major source of lipids). In non egg-yolk
101 containing ASM, the acetate production was significantly reduced compared to standard oASM
102 (at maximum after 8h, levels were at 40 % of levels in oASM, $p<0.02$, T-Test), but was still
103 significantly higher than in fASM (after 8h, levels were 15x higher, $p<0.01$, T-Test). Therefore,
104 we conclude that the egg yolk is an acetogenic substrate in oASM, but is not the only one.
105 These are potentially important differences between the two versions of ASM, given that acetate
106 excretion by *P. aeruginosa* CF clinical isolates has been linked to the length of infection (and
107 hence metabolic adaptation to lung conditions)(1). Formate excretion also differed between the
108 media, with levels in fASM and LB higher than in oASM (Figure 1).

109 In addition to fermentation products, several low concentration metabolites were detectable
110 above baseline after 24 h incubation, and some of these were clearly different between fASM
111 and oASM (data not shown). In-depth characterization of these compounds is beyond the scope

of our current study, but indicates that there could be other specific medium-dependent metabolic differences – which could well be related to cellular signalling, for instance. In summary, direct analysis of changing exometabolomic profiles can highlight bacterial responses to different media; *Pseudomonas aeruginosa* metabolite uptake is broadly comparable across different rich media (and very similar between the filtered and unfiltered versions of ASM), but changes in metabolite excretion indicate that there are also differences in cellular metabolism between media.

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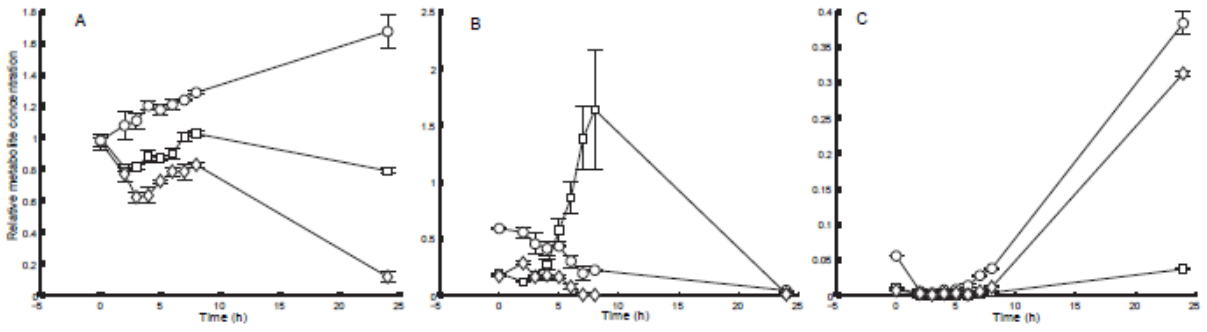
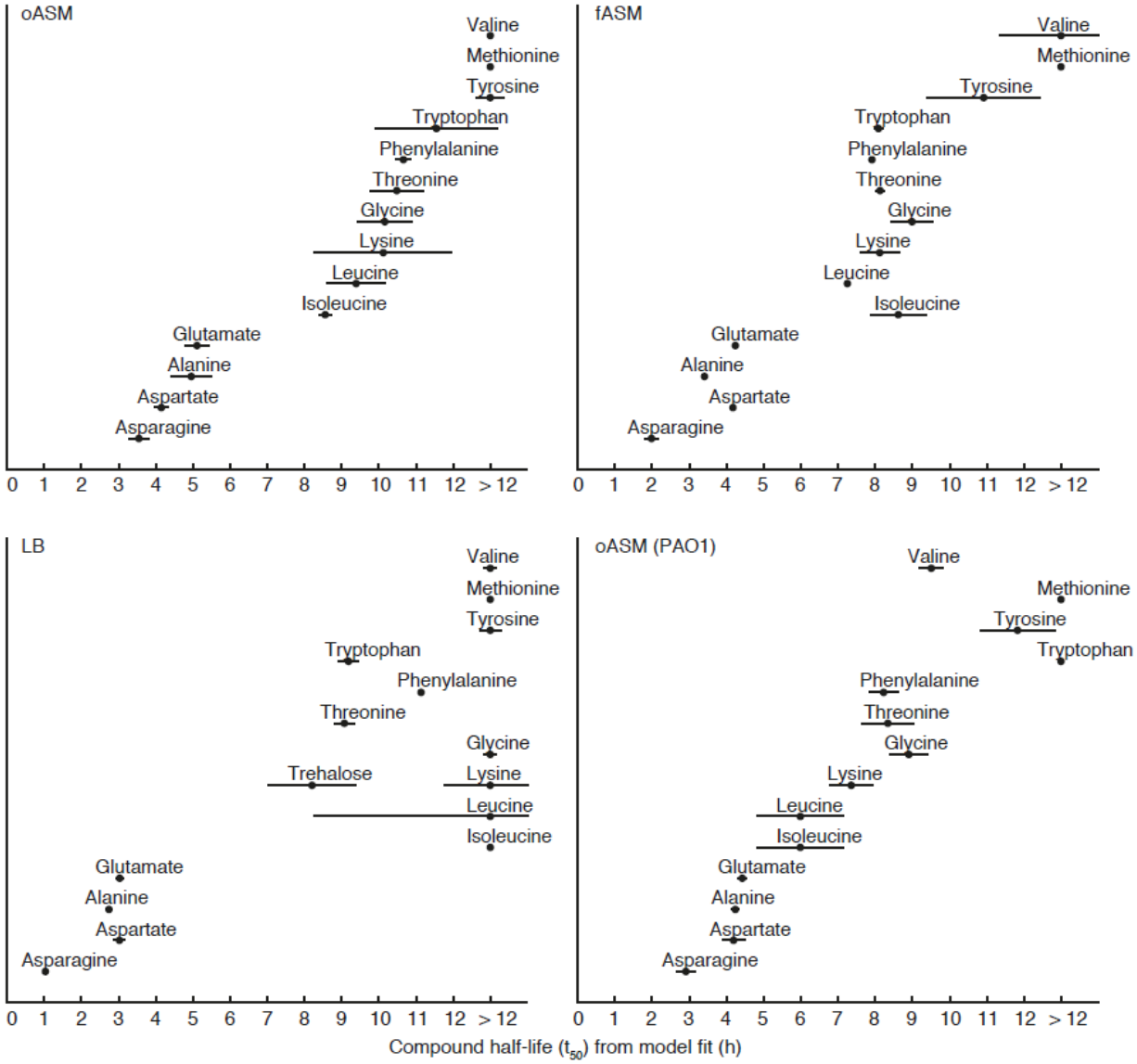
Table 1. Fitted compound half-life (t_{50}) values derived from sigmoid fits of concentration data for metabolites found in all three growth media.

	oASM		fASM		LB		oASM (PA
t_{50}	mean	S.D.	mean	S.D.	mean	S.D.	mean
Asparagine	3.55	0.28	1.98	0.19	1.03	0.03	2.91
Aspartate	4.15	0.20	4.17	0.07	3.02	0.16	4.19
Alanine	4.96	0.56	3.41	0.06	2.74	0.05	4.24
Glutamate	5.11	0.33	4.24	0.03	3.03	0.10	4.42
Isoleucine	8.56	0.18	8.62	0.75	> 12	nd	5.99
Leucine	9.39	0.78	7.25	0.06	> 12	nd	5.99
Lysine	10.12	1.85	8.12	0.54	> 12	nd	7.36
Glycine	10.16	0.73	8.99	0.57	> 12	nd	8.90
Threonine	10.48	0.71	8.13	0.12	9.07	0.28	8.34
Phenylalanine	10.66	0.20	7.91	0.05	11.14	0.00	8.23
Tryptophan	11.55	1.65	8.09	0.12	9.19	0.27	> 12
Tyrosine	> 12	nd	10.92	1.52	> 12	nd	11.83
Methionine	> 12	nd	> 12	nd	> 12	nd	> 12
Valine	> 12	nd	> 12	nd	> 12	nd	9.51




oASM
Valine
Methionine
Tyrosine
Tryptophan
Phenylalanine
Threonine
Glycine
Lysine
Leucine
Isoleucine
Glutamate
Alanine
Aspartate
Asparagine
0 1 2 3 4 5 6 7 8 9 10 11 12 > 12
fASM
Valine
Methionine
Tyrosine
Tryptophan
Phenylalanine
Threonine
Glycine
Lysine
Leucine
Isoleucine
Glutamate
Alanine
Aspartate
Asparagine
0 1 2 3 4 5 6 7 8 9 10 11 12 > 12
LB
Valine
Methionine
Tyrosine
Tryptophan
Phenylalanine
Threonine
Glycine
Trehalose
Lysine
Leucine
Isoleucine
Glutamate
Alanine
Aspartate
Asparagine
0 1 2 3 4 5 6 7 8 9 10 11 12 > 12
oASM (PAO1)
Valine
Methionine
Tyrosine
Tryptophan
Phenylalanine
Threonine
Glycine
Lysine
Leucine
Isoleucine
Glutamate
Alanine
Aspartate
Asparagine
0 1 2 3 4 5 6 7 8 9 10 11 12 > 12
Compound half-life (t_{50}) from model fit (h)

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