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Diversity of metabolic profiles of cystic fibrosis *Pseudomonas aeruginosa* during the early stages of lung infection

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Pseudomonas aeruginosa is the dominant pathogen infecting the airways of cystic fibrosis (CF) patients. During the intermittent colonization phase, *P. aeruginosa* resembles environmental strains but later evolves to the chronic adapted phenotype characterized by resistance to antibiotics and mutations in the global regulator genes *mucA, lasR* and *rpoN*. Our aim was to understand the metabolic changes occurring over time and between niches of the CF airways. By applying Phenotype MicroArrays, we investigated changes in the carbon and nitrogen catabolism of subsequently clonally related mucoid and non-mucoid (NM) lung and sinus *P. aeruginosa* isolates from 10 CF patients (five intermittently colonized/five chronically infected). We found the most pronounced catabolic changes for the early/late NM isolate comparisons, with respiratory reduction seen for all chronically infecting isolates and two intermittently colonizing isolates. Fewer differences were observed between sinus and lung isolates, showing a higher degree of isolate similarity between these two niches. Modest respiratory changes were seen for the early isolate/PAO1 comparisons, indicating colonization with environmental isolates. Assignment of metabolic pathways via the KEGG database showed a prevalence of substrates involved in the metabolism of Ala, Asp and Glu, D-Ala, and Arg and Pro. In conclusion, extensive heterogeneity in the metabolic profiles of the *P. aeruginosa* isolates was observed from the initial stages of the infection, showing a rapid diversification of the bacteria in the heterogeneous environment of the lung. Metabolic reduction seems to be a common trait and therefore an adaptive phenotype, though it can be reached via multiple metabolic pathways.

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INTRODUCTION

The hereditary disease cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane regulator (CFTR), which is a protein regulating the transport of chloride ions across the surface of secretory epithelial cells (Buchanan *et al.*, 2009; Garred *et al.*, 1999). The imbalance in chloride transport across the lung epithelial surface and the impaired detachment of mucus (Brennan *et al.*, 2007; Stoltz *et al.*, 2015) caused by these mutations result in reduced clearance of viscous lung secretions (Brennan *et al.*, 2007), and CF patients are colonized and infected with bacteria such as *Staphylococcus aureus, Haemophilus influenzae* and *Pseudomonas aeruginosa* early in life (Pressler *et al.*, 2006; Rudkjøbing *et al.*, 2012). Over time, *P. aeruginosa* becomes the predominant pathogen

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; M, mucoid; NM, non-mucoid; PM, Phenotype MicroArray; PMN, polymorphonuclear neutrophil; TCA, tricarboxylic acid.

Twelve supplementary tables and three supplementary figures are available with the online Supplementary Material.

infecting both the lungs and paranasal sinuses (Aanaes *et al.*, 2013b; Ciofu *et al.*, 2013) and it is the primary cause of morbidity and mortality in CF (Hansen *et al.*, 2012; Jelsbak *et al.*, 2007; Pressler *et al.*, 2006). The initial focus in CF patients has been shown to be the paranasal sinuses and from this focus the lungs are repeatedly intermittently colonized. Early, aggressive antibiotic eradication therapy of the intermittent lung colonization can prevent subsequent chronic lung infection in most patients, but this therapy fails in about 20 % of patients, who become chronically infected in the lungs (Hansen *et al.*, 2008) with the same clone found in the paranasal sinuses (Folkesson *et al.*, 2012).

P. aeruginosa is a Gram-negative bacterium with the ability to establish infection in various habitats such as soil, water, plants and humans due to its large metabolic versatility (Palmer *et al.*, 2010). The initial intermittent colonization with *P. aeruginosa* in CF patients, where the strains resemble those found in the environment, then over time becomes a chronic biofilm infection with CF airway-adapted strains (Ciofu *et al.*, 2008; Pressler *et al.*, 2006). Mutations in the global regulator genes *mucA*, *lasR* and *rpoN* are selected for in the adaptation phase during the early years (Damkiær *et al.*, 2013; Marvig *et al.*, 2015; Yang *et al.*, 2011) as a response to the stressful conditions encountered by *P. aeruginosa* in the CF lungs (Ciofu *et al.*, 2008; Folkesson *et al.*, 2012). Polymorphonuclear neutrophils (PMNs) and specific antibodies respond to the bacterial infection, but as it is a biofilm infection, it cannot be cleared by the PMNs, instead resulting in lung tissue degradation due to proteolytic enzymes (predominantly elastase) released by PMNs and reactive oxygen species. This eventually causes respiratory failure and either lung transplantation or death in CF patients (Downey *et al.*, 2009; Koch & Høiby, 1993).

P. aeruginosa airway infection is localized in three different anatomical niches with different physiological and immunological conditions: the microphilic or anaerobic paranasal sinuses, dominated by a secretory IgA (s-IgA) response and few PMNs and where the bacteria are kept at a distance from the mucosal epithelial cells by mucus (Aanaes *et al.*, 2011, 2013a; Johansen *et al.*, 2012); the anaerobic sputum inside the conductive zone of the lungs, where both s-IgA and IgG and abundant PMNs originating from the respiratory zone are present and where the bacteria are also kept at a distance from the mucosal epithelial cells by the mucus (Kolpen *et al.*, 2010; Pedersen *et al.*, 1992); and the aerobic respiratory zone, where IgG and PMNs are abundant and where the bacterial biofilms are located next to the epithelial cells or alveoli and the PMNs, which are recruited from the alveolar capillaries (Bjarnsholt *et al.*, 2009; Craig *et al.*, 2009). Due to adaptation to the three different niches and to the intensive antibiotic therapy, different phenotypic subpopulations of the infecting *P. aeruginosa* clone develop (Folkesson *et al.*, 2012; Høiby *et al.*, 2010b).

The CF lung is a heterogeneous environment with microhabitats that develop owing to local differences in the inflammatory reaction between the different focal areas of infection. Studies of bacterial evolution, exemplified by the studies of Rainey and colleagues on *Pseudomonas fluorescens* (Rainey & Travisano, 1998), have demonstrated that bacteria show niche specialization and adaptive radiation when introduced into spatially and chemically heterogeneous environments. We later confirmed this by employing a clinical isolate of *P. aeruginosa* from a CF patient (Hoffmann *et al.*, 2005). The bacteria rapidly evolve into niche specialists to take advantage of this (Maharjan *et al.*, 2007; Rainey & Travisano, 1998). Therefore, while *P. aeruginosa* can be expected to adapt to the changing CF lung environment by regulating gene expression, the complexity of the CF lung may also drive adaptive radiation of the microbial populations from an initial colonizing strain (Behrends *et al.*, 2013). The diversity of colonial morphotypes isolated from patients is supportive of adaptive radiation and niche specialization (Häussler, 2004; Malone *et al.*, 2010; Wahba & Darrell, 1965).

It is important to note that in spite of the apparent parallelism in the phenotypic changes among clinical isolates, there is a high level of phenotypic diversity within an individual patient (Darch *et al.*, 2015; Mowat *et al.*, 2011; Workentine *et al.*, 2013). In spite of this high heterogeneity, parallel changes in bacterial phenotypes have been observed in temporal studies identifying dominant phenotypes within a patient at each time point sampled, and therefore identifying the long-term selective evolutionary events. The chronic, CF-evolved phenotype involves increased antibiotic resistance, reduced virulence, alginate overproduction resulting in mucoid strains forming biofilms, loss of quorum sensing and loss of motility (Ciofu *et al.*, 2010).

Continuous growth of *P. aeruginosa* in the hostile CF lung environment, despite the many adaptive changes undergone by the bacteria, is dependent on nutrient availability. Previous studies have established that the lung mucus is rich in amino acids and that auxotrophy arises during chronic lung infection (Barth & Pitt, 1996; Taylor *et al.*, 1993; Thomas *et al.*, 2000).

In most infection sites, the metabolic pathways important for proliferation of bacterial pathogens are unknown. Basic knowledge regarding metabolic processes utilized by infecting bacteria is of importance for understanding bacterial pathogenesis, and may also offer opportunities for development of novel therapeutics. From a host–pathogen perspective, the carbon sources available for growth significantly affect production of extracellular virulence factors and biofilm formation in *P. aeruginosa* (Klausen *et al.*, 2003; Palmer *et al.*, 2005).

Generally, the nutrition sources for bacteria in the human or animal host are rich, since most bacterial species grow on e.g. broth or broth enriched with serum or on blood or chocolate agar media. There is, however, little insight into the molecular mechanisms of carbon preference or the effects that specific carbon sources have on host colonization and proliferation within infection sites, and

nutrient-versatile bacteria such as *P. aeruginosa* may not need all metabolic pathways during persistent infections, e.g. in CF airways. It is therefore possible that the ongoing adaptation and evolution during chronic infection may lead to loss of metabolic functions that are not needed in the niche of infection (Markussen *et al.*, 2014).

The mean concentrations of ions, free amino acids, glucose and lactate in the sputum from 12 adult CF patients with non-exacerbating *P. aeruginosa* infection were measured by Palmer *et al.* (2007). This study established that the six carbon sources first utilized by *P. aeruginosa* are Pro, Ala, Arg, Glu, Asp and lactate.

It has previously been shown by proteomic and transcriptomic analysis of CF isolates from different stages of the chronic lung infection that adaptation to the lungs involves significant metabolic changes, which lead to variants with increased metabolic fitness in the niches in the CF lungs (Behrends *et al.*, 2013; Hoboth *et al.*, 2009). Based on the study by Yang *et al.* (2011) showing that the major changes occurring in *P. aeruginosa* during chronic CF lung infection happen during the early years of infection, we hypothesized that major metabolic changes would occur in the initial stages of the infection.

In the present study, we have applied Biolog Phenotype MicroArrays (Bochner, 2009) to investigate the metabolic changes in subsequent clonally related isolates from the initial stages of colonization and infection. Thirty-five clinical *P. aeruginosa* isolates from CF patients treated at the Copenhagen CF Centre have been included. The isolates represented the first *P. aeruginosa* isolate followed by subsequent isolates found by culture of sputum or sinus samples. When available, both mucoid (M) and non-mucoid (NM) isolates from lung and sinus were investigated. At the end of the study, six of the patients had become chronically infected, while four were still in the intermittent stage of the colonization. Metabolic profiles of the isolates were compared to each other, to PAO1 and to laboratory mutants harbouring mutations in the global regulator genes *mucA*, *lasR* and *rpoN*.

METHODS

CF patients and clinical isolates. The 10 CF patients included in this study were followed by monthly visits at the Copenhagen CF Center at Rigshospitalet (300 CF patients), where patients are seen once a month for clinical examination, functional lung testing and microbiological examination of lower respiratory secretions as detailed previously (Knudsen *et al.*, 2009). Patients with positive *P. aeruginosa* cultures are treated according to the Copenhagen model, with a combination of inhaled colistin and oral ciprofloxacin whenever *P. aeruginosa* is cultured (Hansen *et al.*, 2008; Johansen *et al.*, 2008). Chronic *P. aeruginosa* lung infection is defined as the presence of *P. aeruginosa* in cultures from the lower respiratory tract continuously for 6 months and or development of fewer than two precipitating antibodies in serum against *P. aeruginosa* (Pressler *et al.*, 2009). To study the metabolic changes occurring in *P. aeruginosa* during the intermittent colonization period, where antibiotic eradication therapy was used to prevent chronic lung infection (Hansen *et al.*, 2008), we selected a series of 35 clinical isolates from 10 CF patients (Table 1) taken during the intermittent colonization period: 'early' isolates, and either after repeated successful antibiotic eradication ('late intermittent isolates') in five patients (CF405, CF408, CF414, CF430 and CF431) or immediately after the establishment of chronic lung infection ('late chronic isolates') in five patients (CF312, CF341, CF483, CF519 and CF544). All of the early isolates were obtained from CF patients that fulfilled the criterion of fewer than two precipitating antibodies present at the time of isolation and should thus be considered intermittently colonized (Johansen *et al.*, 2004), except for CF483. Infection status, CFTR mutation and gender of the 10 patients are listed in Table S1 (available in the online Supplementary Material).

PFGE was used for genotyping of each of the clinical isolates. The clinical isolates included in this study for each patient were chosen based on matching PFGE patterns (clonally related) allowing up to three different bands between isolates from the same patient (Tenover *et al.*, 1995). No strain was shared among the 10 CF patients included in the study.

The clinical CF isolates were genotypically (sequencing of the global regulators *mucA*, *algT*, *lasR* and *rpoN*) and phenotypically (antibiotic susceptibility, mutation frequency, protease activity, colony morphology, screening for possible auxotrophs and *in vitro* growth rates) characterized as previously described (Ciofu *et al.*, 2005, 2010; Yang *et al.*, 2011).

The sinus isolates were collected by functional endoscopic sinus surgery (FESS) as previously described (Johansen *et al.*, 2012). The sinus isolates included in the study were obtained from the left and right sinus maxillaries during FESS.

The lung samples (sputum) were Gram-stained and examined microscopically to confirm the lower airway origin of the samples. Both sputum and sinus samples were plated on blue agar plates, a modified Conrad Drigalski medium selective for Gram-negative rods as described by Høiby (1974) (SSI Diagnostica). *P. aeruginosa* colonies from the blue plates were collected and frozen. The selection criteria were phenotypic (M and NM), and different patterns of resistance to antibiotics as determined on the basis of direct sensitivity testing of isolates from plated sputum (Ciofu *et al.*, 2010).

M and/or NM *P. aeruginosa* isolates were collected and stored at −80 °C in serum broth with 10 % glycerol as a cryoprotectant (SSI Diagnostica). Both an M and NM *P. aeruginosa* isolate simultaneously present in the sputum samples were considered an isolate pair (Ciofu *et al.*, 2008).

Heterogeneity of sputum samples. To assess the heterogeneity of the sputum bacterial populations, sputum samples from CF341, CF405 and CF430 were collected, and 33, 96 and 20 colonies, respectively, were tested for antibiotic susceptibility towards ciprofloxacin, ceftazidime, meropenem and colistin by Etest. Colony morphology was assessed by microscopy. The possible auxotrophy of the colonies was tested by replica plating the overnight cultures of each colony to both glucose minimal medium and LB. Possible auxotrophs were scored as cells forming colonies on the LB plates, but not on minimal medium plates, after 48 h of incubation at 37 °C.

***P. aeruginosa* mutants.** All laboratory mutants had a *P. aeruginosa* PAO1 background. PAO1 was originally isolated from an Australian burn wound patient (Holloway, 1955). *mucA*: PDO300 is a mucA22 mutant constructed as described by Mathee *et al.* (1999). The *lasR* mutant was constructed as described by Rasmussen *et al.* (2005). The *rpoN* mutant was kindly provided by Søren Damkiær (Department of Systems Biology, Technical University of Denmark) and constructed as described by Damkiær *et al.* (2013).

Table 1. Analysed CF lung and sinus isolates

The sinus isolates are obtained from different parts of the paranasal sinuses M, Mucoid; NM, non-mucoid; P, number of *P. aeruginosa* precipitin; po, peroral; inh, inhalation; IV, intravenous.

Final infection status	Isolate	M/NM	From	Year of isolation	Pp	Infection stage* (years before (−)/after (+) chronic infection)	Antibiotic therapy at isolate collection time (weeks)
Intermittently colonized	CF405-56281A	NM	Lung (early)	2005	1	Intermittent	po: 4; inh: 8
	CF405-240A	NM	Sinus	2008	0	Intermittent	po: 50; inh: 60; IV: 4§
	CF405-240B	M	Sinus	2008	0	Intermittent	po: 50; inh: 60; IV: 4§
	CF405-3299	NM	Lung (late)	2010	2	Intermittent	po: 65; inh: 80; IV: 10
	CF408†-19970	NM	Lung (early)	2006	0	−5	0
	CF408-SP05	M	Lung	2007	1	−4	po: 26; inh: 38; IV: 4§
	CF408-SP13	NM	Lung	2007	1	−4	po: 26; inh: 38; IV: 4§
	CF408-38574A	NM	Lung (late)	2008	0	−3	po: 50; inh: 64; IV: 4§
	CF408-SP02	NM	Sinus	2008	0	−3	po: 50; inh: 64; IV: 4§
	CF414-15231	NM	Lung (early)	2006	0	Intermittent	0
	CF414-232	NM	Sinus	2008	0	Intermittent	po: 76; inh: 90
	CF414-18275	NM	Lung	2008	0	Intermittent	po: 3: inh: 3
	CF414-36687	NM	Lung (late)	2009	2	Intermittent	po: 65; inh: 65; IV: 8
	CF430-11621	NM	Lung (early)	2006	0	Intermittent	po: 1
	CF430-142	NM	Sinus	2007	0	Intermittent	po: 62; inh: 63; IV: 7
	CF431-24293	NM	Lung (early)	2008	2	Intermittent	0
	CF431-680	NM	Sinus	2011	2	Intermittent	po: 69; inh: 62; IV: 10
	CF431-7494	NM	Lung (late)	2011	2	Intermittent	po: 39; inh: 39; IV: 4
Chronically infected	CF312-16704	NM	Lung (early)	1991	0	−3	0
	CF312-20441A	M	Lung	1995	9	+1	po: 64; inh: 50; IV: 6§
	CF312-20441B	NM	Lung (late)	1995	9	+1	po: 64; inh: 50; IV: 6§
	CF341-28897	NM	Lung (early)	2006	1	−1	0
	CF341-43885A	M	Lung	2008	28	+1	po/inh: 6; IV: 14
	CF341-43885B	NM	Lung (late)	2008	28	+1	po/inh: 6; IV: 14
	CF341-25907	M	Lung	2010	31	+3	po: 53; inh: 40; IV: 14
	CF341-554	M	Sinus	2010	31	+3	po: 53; inh: 40; IV: 14
	CF483-7161A	NM	Lung (early)	2004	4	0	po: 14; inh: 12; iv: 2
	CF483-2728A	NM	Lung (late)	2006	16	+2	po: 52; IV: 7§
	CF483-2728B	M	Lung	2006	16	+2	po: 52; IV: 7§
	CF519-19637B	NM	Lung (early)	2007	2	0	0
	CF519-17006-1	M	Lung	2011	7	+4	po: 60; inh: 79, IV: 14§
	CF519-17006-2	NM	Lung (late)	2011	7	+4	po: 60; inh: 79; IV: 14§
	CF544-55286B	NM	Lung (early)	2010	1	−1	0
	CF544-60163A	M	Lung	2011	8	0	po: 24; inh: 24; IV: 8§
	CF544-60163B	NM	Lung (late)	2011	8	0	po: 24; inh: 24; IV: 8§

*Intermittent colonization or years before (−)/after (+) the establishment of chronic infection.

†CF408 became chronically infected after conclusion of the study.

‡*P. aeruginosa*-free November 2005 to October 2006.

§The M/NM pairs and the two CF408 NM isolates, respectively, were collected on the same days.

***P. aeruginosa* environmental isolates.** To assess the heterogeneity of the catabolic profiles of different *P. aeruginosa* strains, five environmental strains isolated from sewage (PS7, PS21, PScol11, PSF8 and PSM4) were analysed and compared to PAO1. These five strains are part of the Lindberg phage typing set (Lindberg & Latta, 1974).

Phenotype MicroArrays. Phenotype MicroArrays (PMs) for microbial cells (Biolog) were applied to create metabolic profiles of selected *P. aeruginosa* isolates (clinical isolates and laboratory strains). The isolates were tested in duplicates on three different microtitre plates; on PM1 and PM2 containing 95 different carbon sources each, and on PM3 containing 95 different nitrogen sources. The bacterial inocula were prepared according to the manufacturer's protocol for Gram-negative bacteria. The strains were grown on blue agar plates selective for Gram-negative bacteria (SSI Diagnostica) overnight or until sufficient amounts of bacteria were present depending on fast- or slow-growing phenotypes. A dense solution of the bacteria was creating by scraping the cells off the plates and dissolving them completely in approximately 600 µl inoculum fluid (IF-0a GN/GP base 1.2 ×; Biolog). This solution was then used to set the turbidity to 42 % using a turbidimeter (Biolog) by adding the suspended bacteria gradually to 10 ml pure inoculum fluid and dissolving completely. The high cell count was applied to limit the growth potential for the faster-growing bacteria allowing for the same metabolic conditions for both fast- and slow-growing strains. The bacterial suspension was mixed with inoculum fluid, water and a yellow tetrazolium dye (Biolog Redox Dye A 100 ×) to report metabolic activity. For PM3, a 2 M sodium succinate/200 µM ferric citrate solution was added as a carbon source, reaching a final concentration of 20 mM sodium succinate/2 µM ferric citrate in the inoculated suspension. Inoculum (100 µl) was added to each well and the plates were incubated in the Omnilog plate reader (Biolog) for 72 h at 37 °C, with the measurement of colour change from yellow to purple as an indicator of bacterial respiration performed every 15 min. This created respiration curves for each tested strain, which were analysed using the Omnilog-OL_PM_FM/Kin 1.20.02 and Omnilog-OL_PM_Par 1.20.02 software programs supplied by Biolog. The parameter used for the statistical analysis was the area under the respiration curves.

Selection of significant substrates

Statistical analysis. For the statistical analysis, the normal variation in each well was determined by repeated testing of our reference strain PAO1 eight times for 72 h.

All test strains were determined in duplicates. In the statistical test below, the result of a double determination was accepted (no significant difference between the two determinations) if the test revealed a value of less than two:

$$|t_1 - t_2|/(\sqrt{2} \times \mathrm{SD_P}) < 2 \quad (1)$$

In this equation, t_1 and t_2 are the areas under the respiration curves of the two determinations, respectively, and $\mathrm{SD_P}$ is the SD of the areas under the curves of the eight determinations of the respiration of the reference strain PAO1.

To determine if one strain was significantly different from another strain, a second test was performed:

$$|\overline{X} - \overline{Y}|/\mathrm{SD_P} < 2 \quad (2)$$

In this equation, $\overline{X}$ is the mean of the first double determination, $\overline{Y}$ is the mean of the second double determination and $\mathrm{SD_P}$ is the SD of the areas under the respiration curves of the eight determinations of the reference strain PAO1, as in equation 1. By performing this test, we ensured that two strains were found to be significantly different only if the difference between the two strains compared was larger than the normal variation observed for PAO1 in the given well.

Informative substrates. Based on eight 72 h determinations of our reference PAO1 strain (MH340), we set up parameters for which wells to include in our study. We apply the term 'substrate' for the names of chemicals and the term 'source' when grouping substrates as sources of carbon or nitrogen.

For the carbon sources (PM1 and PM2), wells were included if the slope of the 72 h respiration curve of PAO1 was larger than two. For the nitrogen sources (PM3), only wells with respiration earlier than 24 h of incubation were included, as significant reduction of the tetrazolium dye was observed after approximately 24 h in the negative control (A01). These wells were selected by the area under the respiration curves; only wells with an area larger than that of the background respiration in the negative control were included. This was to ensure that the respiration analysed was due to the utilization of the coated substrate.

Furthermore, as the PMs generate a vast amount of data, we limited our analysis to the substrates involved in the central metabolism of *P. aeruginosa* listed in the KEGG database (http://www.genome.jp/kegg/). For pathway assignments, we assigned substrates to the tricarboxylic acid (TCA) cycle and amino acid metabolisms.

We thus included 28 carbon sources on PM1, 27 carbon sources on PM2 and 50 nitrogen sources on PM3. Full lists of the included substrates are found in Tables S2 and S3.

Biological differences. In addition to the statistical analysis and the selection of substrates found to be informative, we excluded substrates on which the difference in areas of the two compared respiration curves was less than 20 000 'software units'. This was to ensure the inclusion of only substrates displaying biological differences in respiration of the compared strains.

Definition of respiratory responses. For the data analysis, respiratory reduction was defined as the impaired respiration of the test strain compared to the reference strain, when the area of the respiration curve of the reference strain was >20 000 'software units' larger than the area of the respiration curve of the test strain. Respiratory improvement defined a better respiration of the test strain compared to the reference strain, when the area of the respiration curve of the reference strain was >20 000 'software units' smaller than the area of the respiration curve of the test strain.

Catabolic profiles. To characterize the catabolic changes of the isolate pairs, we assigned metabolic pathways to the selected substrates using the KEGG database (http://www.genome.jp/kegg/), choosing *P. aeruginosa* PAO1 as the organism for the pathways. If two or more substrates were part of the same pathway, their positions in the given pathways were mapped. Due to the comprehensiveness of the KEGG metabolic maps, only the pathways where our selected substrates were directly connected, and part of the actual pathway rather than links to other pathways, were further analysed. The changes in the respiratory response were viewed per patient and later compared between patients by involvement in selected pathways.

Ethics. Informed consent was obtained from all patients.

RESULTS

Phenotypic and genotypic characteristics of the *P. aeruginosa* isolates

An overview of the genotypic and phenotypic characteristics of the 35 clinical isolates included in this study is

presented in Tables S4 and S5, respectively, and the phenotypic characteristics of the laboratory mutants are presented in Table S6.

Heterogeneity of the sputum bacterial populations as assessed by antibiotic sensitivity, colony morphology and possible auxotrophy showed a more homogeneous bacterial population in the sputum samples of the CF patients that were intermittently colonized, compared to the chronically infected patients (Fig. S1).

Phenotype MicroArrays data

Metabolic profiles of laboratory and clinical CF *P. aeruginosa* isolates were created by applying PMs for microbial cells.

Comparison of PAO1 with environmental and early CF lung isolates. The earliest available lung isolates from each of the 10 selected patients were compared to PAO1, to investigate the catabolic changes occurring at this infection stage. *P. aeruginosa* isolates from eight of the 10 patients showed very modest changes, primarily respiratory reduction, on few (up to four) carbon sources. Respiratory reduction on a large number of carbon sources (34 and 25, respectively) was seen for the *P. aeruginosa* isolates from two patients, one who remained intermittently colonized throughout the study (CF430) and one who subsequently became chronically infected (CF544), when compared to PAO1. All the substrates showing respiratory reduction for this comparison are listed in Table S7. To determine if the metabolic differences seen in the early CF isolates when compared to PAO1 were due to early adaptation in the CF lung, or were a consequence of infection with different environmental *P. aeruginosa* strains, we performed PM analysis on five environmental strains isolated from sewage (PS7, PS21, PScol11, PSF8 and PSM4) and compared these to PAO1. Loss of phenotype compared to PAO1 was mainly observed, as only three of the environmental isolates showed gain of phenotype of a single carbon source, the amino acid D-Ala (Table 2).

We hypothesized that changes found only in the clinical isolates, and not in the environmental isolates, would reflect possible adaptive metabolic changes. Therefore, the metabolic changes of the environmental isolates were compared to those observed for the early CF isolates. Table 2 lists the substrates common for two or more early CF isolates and one or more environmental isolates. Reduction of respiration on pyruvate and 4-hydroxybenzoate was observed exclusively in clinical isolates.

Table 2. Respiratory changes for the early CF isolates compared to PAO1 and the environmental PS isolates compared to PAO1

Only the substrates shared by two or more CF isolates or environmental isolates are included.

Substrates	Early CF isolates										Environmental isolates (PS)				
	Intermittently colonized					Chronically infected									
	CF405	CF408	CF414	CF430*	CF431	CF312	CF341	CF483	CF519	CF544*	7	21	col11	F8	M4
Reduction (C)															
4-Hydroxybenzoate				✓				✓	✓			✓	✓		
Acetate				✓						✓					
β-Hydroxybutyrate				✓						✓	✓				
D-Arabitol	✓					✓				✓		✓			
Fumarate				✓						✓	✓				
Inosine		✓		✓	✓					✓	✓				
L-Arg				✓						✓		✓			
L-Leu				✓			✓			✓	✓				
N-Acetyl-L-Glu				✓				✓	✓	✓	✓				
Pyruvate	✓			✓	✓										
Quinate				✓			✓			✓	✓	✓			
Succinate				✓						✓	✓	✓	✓		✓
Improvement (C)															
D-Ala		✓	✓									✓	✓		✓
Reduction (N)															
β-Phenylethylamine	✓	✓		✓	✓	✓					✓	✓			
Tyramine		✓		✓									✓		

*Only the substrates common for CF430 and CF544 and one or more other CF isolate and/or environmental isolate are listed. (C), Carbon source; (N), nitrogen source.

Comparison of early and late NM lung isolates. PM analysis was performed on NM paired isolates taken before (early isolates) and after the establishment of chronic infection or at a later stage of the intermittent colonization (late isolates), from nine of the ten selected CF patients (Table 1). The respiration curves of the compared early and late isolates on PM1 and PM2 are shown in Figs S2 and S3. When examining the metabolic differences between isolates obtained at different time points for the patient, we view the patients according to their infection status.

Changes on carbon sources of the late NM compared to the early NM isolates. Comparison of the early and late NM isolates showed primarily respiratory reduction in the late isolate. Two of the four intermittently colonized patients had respiratory reduction on 16 and 24 substrates, respectively, and all the late isolates from the chronically infected patients showed respiratory reduction on between 1 and 12 substrates (Table 3). Interestingly, one of the two intermittently colonized patients with respiratory reduction on several substrates (CF408) has later become chronically infected.

Using the KEGG database, we made pathway assignments if two or more substrates were directly connected to each other in the same pathway (Table 4). The pathways were Ala, Asp and Glu metabolism; Arg and Pro metabolism; D-Ala metabolism; D-Gln and D-Glu metabolism; the TCA cycle; and the fructose and mannose metabolism. Table S8 and Figs 1 and 2 show which substrates are involved in the given pathways and which of these are directly connected. The observed respiratory improvement is shown in Table S9.

For the isolates from intermittently colonized patients, pathways were assigned to the early/late isolate comparisons for CF408 and CF414 (Tables 3 and 4a). Directly connected substrates were seen in three amino acid metabolisms, and the fructose and mannose metabolism, as described in detail in Table 8S and Figs 1 and 2.

For the chronically infected patients, directly connected substrates were seen for two of the five patients in the TCA cycle and in three amino acid metabolisms (Tables 3 and 4a). A detailed description of how the substrates are connected in the pathways is found in Table S8 and Fig. 1.

Changes on nitrogen sources of the late NM compared to the early NM isolates. The respiratory reduction on nitrogen sources is shown in Table 3 and the common pathways are listed in Table 5. Two substrates were found to be connected in the Phe, Tyr and Trp metabolic pathway for both isolate comparisons listed here (Fig. 3). A full list of the nitrogen sources showing respiratory reduction in all the early/late isolate comparisons is found in Tables 3 and S10.

Mucoid/NM lung and sinus isolates. M/NM isolate pairs collected at the same time from the same sample were available for all five chronically infected patients and two intermittently colonized patients (CF408 from a sputum sample and CF405 from a sinus sample). The metabolic profile of the PAO1 *mucA* mutant is shown in Table S11.

Respiratory reduction for M compared to NM isolates was observed in three out of the seven pairs of isolates (Table 6). Pathways were assigned for two of them (Tables 4b and S8).

The sinus M isolates from the intermittently colonized CF405 showed increased respiration on the carbon source D-Ala involved in D-Ala metabolism, compared to the NM isolate (Table S9).

Changes on nitrogen sources are shown in Table 6. No direct connection between substrates in any metabolic pathway was found for either of the isolate pairs.

Comparison of lung and sinus CF isolates. Differences in the catabolic profiles between sinus isolates sampled from different sinus locations were found, showing distinct adaptive pathways in the various areas of the sinuses (data not shown). The lung–sinus comparison yielding the largest metabolic difference was used for further analysis.

For all the lung–sinus pairs, pathways were assigned to the carbon sources, which showed respiratory reduction and respiratory improvement (Table 7).

The assignment of metabolic pathways to the carbon sources revealed fewer pathways with directly connected substrates than for the early/late and M/NM isolate pairs (Tables 4c and S8). Metabolic improvement on D-Ala (D-Ala metabolism) and D-fructose and D-mannitol (fructose and mannose metabolism) was seen in sinus compared to lung isolates (Tables 4c and 7).

The respiratory changes on nitrogen sources seen for the sinus isolates compared to lung isolates are shown in Table 7. No direct connection between any substrates was found.

DISCUSSION

In this study, we have characterized the catabolic profiles of *P. aeruginosa* from 10 CF patients, in an attempt to understand the metabolic changes that occur over time and between different niches of the airways. Based on a unique collection of *P. aeruginosa* isolates representing different stages of infection ('early' and 'late') as well as different niches of the airways (lung and sinus) and different phenotypes (M and NM), we have characterized the associated catabolic profiles and metabolic changes of *P. aeruginosa*. The time between the early and late isolates covers a period of up to six years which, according to our previous estimates of *in vivo* growth rates of *P. aeruginosa* in CF patients, is up to about 38 000 generations (Yang *et al.*, 2008). Our hypothesis was that major adaptive

Table 3. Carbon and nitrogen sources showing respiratory reduction in the comparison of early and late NM isolates

Intermittent					Chronic				
CF405	**CF408**	**CF414**		**CF431**	**CF312**	**CF341**	**CF483**	**CF519**	**CF544**
		Carbon					**Carbon**		
No change	D-Ala	D-Ala	L-Malate	No change	L-Ala	Acetate	L-His	L-Ala	D-Mannitol
	L-Ala	L-Ala	Propanoate		L-Arg	Inosine	L-Hydroxy-Pro α-KG	L-His	
	L-Arg	L-Arg	β-Hydroxybutyrate		L-Asn	Malonate		L-Ile	
	L-Asp	L-Asp	δ-Aminovalerate		L-Asp			L-Leu	
	L-His	L-Glu	Inosine		L-Glu			Pyruvate	
	L-Ile	L-Gln	Quinate		L-Gln			D-Arabitol	
	N-Acetyl-L-Glu	L-Ile	Tyramine		L-His			D-Mannitol	
	Tyramine	L-Pro			α-KG			D-Ribose	
	4-Hydroxy-benzoate	Succinate			L-Hydroxy-Pro			Inosine	
		α-D-Glucose			*N*-Acetyl-L-Glu				
	Glycerol	D-Fructose			Succinate Fumarate				
	D-Arabitol	D-Gluconate							
	D-Fructose	Glycerol							
	D-Mannitol	Acetate							
	δ-Aminovalerate	D-Mannitol							
		D-Ribose							
	Propanoate	L-Lactate							
		Nitrogen					**Nitrogen**		
No change	L-Met	L-Ile	Ammonia	No change	49 Nitrogen sources, see supplementary table 10	No change	No change	No change	No change
	L-Tyr	L-Phe							
		L-Trp							
		L-Tyr							

Table 4. Pathways common to the selected carbon sources in the comparisons of the early/late M/NM lung isolate pairs and the lung/sinus isolate pairs sorted by pathway type and name.

The pathways are sorted by class according to the KEGG database (http://www.genome.jp/kegg/).

Pathway	(a) Early (r)/late (t)* NM lung				(b) NM(r) M(t) lung		(c) Lung (r)/sinus (t)		
	Reduction				Reduction		Reduction	Improvement	
	CF408	CF414	CF312	CF519	CF312	CF341	CF414	CF408	CF430
Carbohydrate metabolism									
Citric acid cycle			✓†		✓				
Amino acid metabolism									
Ala, Asp and Glu		✓	✓	✓	✓	✓			
Arg and Pro			✓		✓		✓		
Val, Leu and Ile‡					✓				
D-Ala	✓	✓							✓
D-Gln and D-Glu		✓	✓						
Other									
Fructose and mannose	✓					✓		✓	✓

*r, Reference strain; t, test strain.
†✓, Two or more substrates are directly connected.
‡Val, Leu and Ile biosynthesis.

metabolic changes may occur in the initial stages of the CF lung infection.

PMs have previously been used to determine the evolutionary dynamics of *P. aeruginosa* isolates from CF patients (Feliziani *et al.*, 2014; Markussen *et al.*, 2014; Yang *et al.*, 2011) and to determine growth and virulence traits (Oberhardt *et al.*, 2008), but we are, to the best of our knowledge, the first to use this method to assign specific metabolic pathways to selected substrates in order to investigate the metabolic adaptation patterns of *P. aeruginosa* isolates from CF patients.

In the present study, which included isolates from an earlier stage of the colonization and infection than our previous publication (Yang *et al.*, 2011), catabolic changes in the form of respiratory reduction were observed more frequently in isolates from chronically infected patients (five out of five) than in isolates from intermittently colonized patients (two out of four), although one of the latter became chronically infected. This is in accordance with previous studies reporting metabolic reduction (Behrends *et al.*, 2013; Feliziani *et al.*, 2014; Markussen *et al.*, 2014; Yang *et al.*, 2011).

In accordance with studies reporting the presence of amino acids in CF sputum and the ability of *P. aeruginosa* to utilize these for growth (Barth & Pitt, 1996; Palmer *et al.*, 2007; Thomas *et al.*, 2000) the primary carbon sources found to show respiratory reduction during the infection and in the lung compared to sinus are amino acids, carbohydrates and carboxylates, and we therefore chose to include only these for further analysis.

Heterogeneity in the metabolic profiles of PAO1 and the first isolates was found, irrespective of the later course of the infection (intermittent or chronic) (Table S7). Respiratory reduction of M compared to NM isolates on carbon and nitrogen sources was seen mainly for the chronically infected patients, and involved primarily Ala, Asp and Glu metabolism (Tables 4 and 6). No major parallel catabolic changes could be observed between the M and NM paired isolates, suggesting a high heterogeneity of these phenotypes.

As previously shown, reduction of metabolic capacity seems to be a fitness peak and an adaptive change, but the pathways used to achieve this seem to vary among isolates, as has been shown by metabolomics studies by Behrends *et al.* (2013). Thus, the differences in the catabolic profiles of the isolates are probably shaped by the heterogeneous environment in the CF lung that occurs as a consequence of the selective pressures the bacteria encounter in the different niches. Using PMs to track common metabolic pathways affected in *P. aeruginosa* during the adaptation to the CF lung would require analysis of a larger number of isolates from the same sputum sample. However, our analysis revealed some common trends.

The vast majority of substrates affected in the isolate comparisons were not linked in any KEGG metabolic pathway. However, we cannot rule out that the observed changes do in fact have implications for the bacterial metabolic

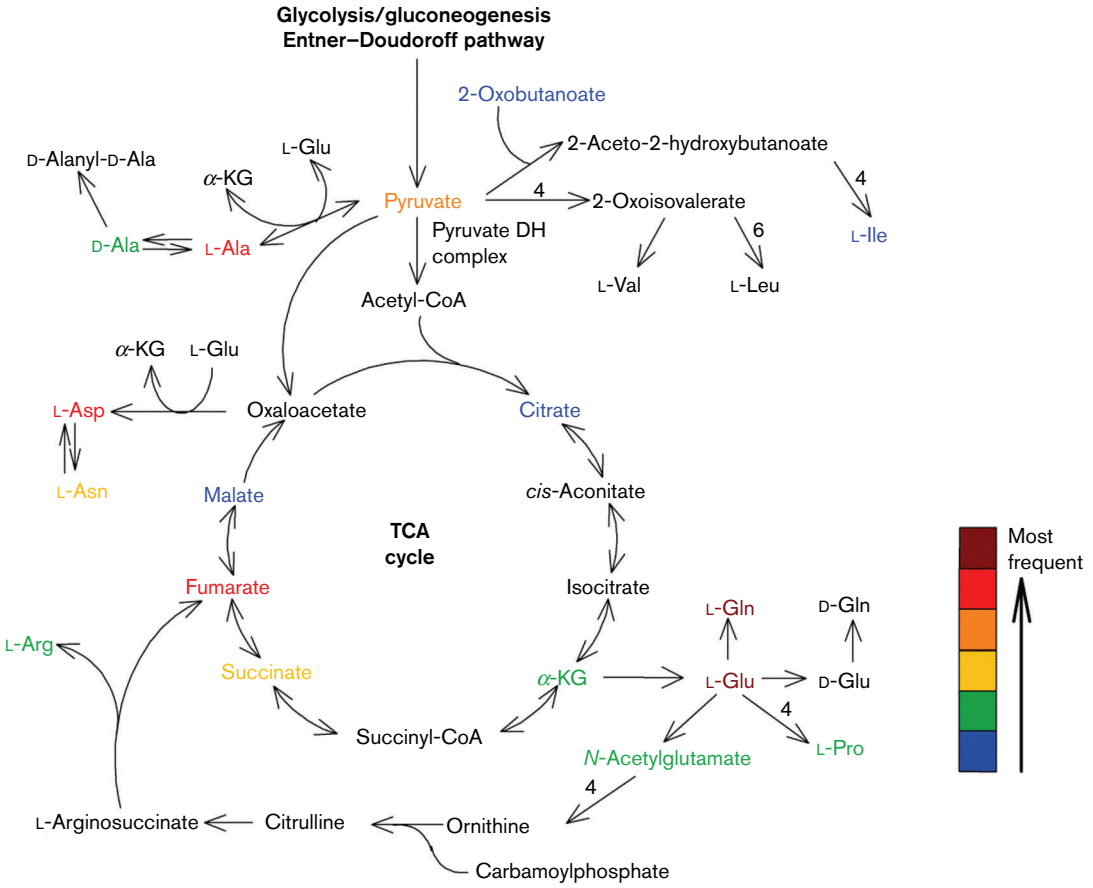

Fig. 1. The TCA cycle and biosynthesis of the amino acids listed in Table 4. Numbers above arrows indicate the number of unincluded intermediate reactions. Selected substrates are highlighted according to how frequently they are listed as a carbon source showing respiratory reduction in Table S8. α-KG: α-ketoglutarate; 2-oxobutanoate: α-ketobutyrate; DH, dehydrogenase. The figure is constructed from data from the KEGG database (http://www.genome.jp/kegg/) and the PseudoCyc database (www.pseudomonas.com:1555/).

response *in vivo*. The metabolic maps from the KEGG database provide a vast amount of information, and in order to give an overview of the pathway reactions we have mapped, Figs 2 and 3 were constructed using information from the KEGG database as well as the PseudoCyc database. Pyruvate, α-ketoglutarate (α-KG), oxaloacetate and L-Glu are central to carbon metabolism, as L-Ala, L-Asp and L-Glu are formed via transamination of pyruvate, oxaloacetate and α-KG, respectively; L-Gln and L-Asn are formed from L-Glu and L-Asp, respectively; and L-Pro and L-Arg are formed from L-Glu. Several isolates show respiratory reduction on one or more of these substrates (Tables 2, 3, 6 and 7; Fig. 1).

The KEGG pathway affected for most of the late isolates is Ala, Asp and Glu metabolism, which involves several substrates showing respiratory reduction in our analysis (Tables S8 and S10). The D-Ala metabolism as shown in the KEGG database for *P. aeruginosa* PAO1 involves only three substrates in two reactions; the conversion of L-Ala to D-Ala, and the conversion of D-Ala to D-alanyl-D-Ala involved in peptidoglycan metabolism. This pathway is therefore likely affected by the reduced respiration on D-Ala and L-Ala in many late CF isolates (Table 5). It has been shown that *P. aeruginosa* catabolizes L-Ala over other carbon sources *in vitro*, and that it is a preferred *in vivo* carbon source (Boulette *et al.*, 2009). Our findings of loss of this pathway in late isolates suggest that Ala plays an important role only in the initial stages of the infection, and not once the infection is established.

Some metabolic pathways (Ala, Asp and Glu metabolism, D-Ala metabolism, D-Gln and D-Glu metabolism and Arg and Pro metabolism) have been found to be more frequently involved in the catabolic changes identified during colonization of the CF airways (Table 4). As shown by Palmer *et al.* (2007), *P. aeruginosa* preferentially catabolizes L-Ala, L-Arg and L-Glu over other carbon sources from CF sputum, which is in agreement with our data, as these substrates are found among the shared lost carbon sources in most isolate comparisons.

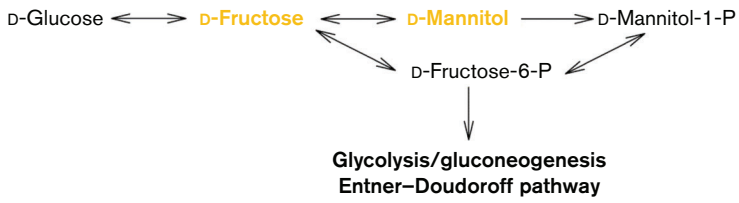

Fig. 2. The connection of D-fructose and D-mannitol in the fructose and mannose metabolism. P, phosphate. Two substrates are highlighted according to how frequently they are listed as a carbon source showing respiratory reduction in Table S8, as in Fig. 1. The figure is constructed from data from the KEGG database (http://www.genome.jp/kegg/) and the PseudoCyc database (www.pseudomonas.com:1555/).

Compared to the early and late lung isolates, differences in a more limited number of substrates, as well as fewer common pathways, were observed between sinus and lung isolates, showing a higher degree of similarity between the isolates from the two niches. In addition, improved respiration in sinus compared to lung isolates was also observed. This suggests, as previously reported (Hansen *et al.*, 2012), that *P. aeruginosa* colonizes the sinuses before migration into the lungs (Fothergill *et al.*, 2014).

Interestingly, analysis of sinus isolates from the right and left sinus maxillaries of several patients revealed differences in the catabolic profiles (data not shown), suggesting different adaptive metabolic pathways in the right and left sinuses. We cannot however rule out the possibility that the heterogeneity observed within the bacterial populations in the paranasal sinuses might account for the observed differences between sides. However, previously published results from our group (Hansen *et al.*, 2012) showed the presence of different morphotypes in the left and right paranasal sinuses in intermittently colonized patients. Little is known about the nutrient composition of the mucus in the paranasal sinuses, although due to the similarity of the mucosal cells in the sinuses and lung, a similar composition might be expected. Due to the small dimensions of the ostium, drainage in the nasal cavity is impaired in CF, and chronic sinusitis is therefore found in nearly all patients. It has been shown that microaerophilic conditions are encountered (Aanaes *et al.*, 2011), that fewer PMNs are present surrounding the bacteria (Johansen *et al.*, 2012) and that lower antibiotic concentrations are achieved in the sinus compared to the lung, since the nebulized antibiotics do not reach the paranasal sinuses (Moeller et al., 2009).

Table 5. Pathways common for the selected nitrogen sources in the comparisons of the early/late isolate pairs as for the carbon sources

Nitrogen sources	Early (r)/late (t)* NM lung	
	Reduction	
Pathway	**CF414**	**CF312**
Amino acid metabolism		
Ala, Asp and Glu		✓†
Arg and Pro		✓
D-Ala		✓
D-Gln and D-Glu		✓
Gly, Ser and Thr		✓
Phe, Tyr and Trp	✓	✓

*r, Reference strain; t, test strain.
†✓, Direct connection of two or more substrates in the pathway.

We observed no particular differences in the respiratory changes between PAO1 and the early isolates in four out of five CF patients in either of the two patient groups: those that subsequently remained intermittently colonized or those who became chronically infected. This similarity between early CF lung isolates and environmental *P. aeruginosa* isolates has been shown previously (Burns *et al.*, 2001). The lack of difference between strains collected from CF patients that followed different courses of lung infection is in accordance with another study, where some phenotypic and genotypic properties were studied on isolates from intermittently colonized CF patients. Here, some patients got rid of the intermittent *P. aeruginosa* colonization whereas others became chronically infected, and no differences were found between the isolates from the two groups of patients (Tramper-Stranders *et al.*, 2012).

Metabolic changes in the early isolates compared to PAO1 were observed in the early isolates from only one patient in each group. These metabolic differences are likely explained by colonization with different environmental *P. aeruginosa* isolates (Table S7) (Hogardt & Heesemann, 2010). However, the metabolic differences between the isolates, in terms of difference in area of the respiration curves (Figs S2 and S3), are relatively small, which indicates respiration of both the early CF isolates and the environmental isolates on the substrates in question despite the phenotypic loss. The metabolic versatility of *Pseudomonas* makes it able to assimilate a wide number of compounds. *P. aeruginosa* preferentially utilizes TCA cycle intermediates, such as the C_4-dicarboxylates

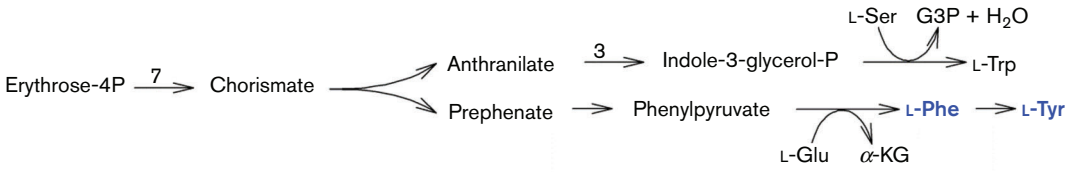

Fig. 3. The connection of L-Phe and L-Tyr in the Phe, Tyr and Trp metabolic pathway. Two substrates are highlighted according to how frequently they are listed as a nitrogen source showing respiratory reduction in Table S10, as in Fig. 1. The figure is constructed from data from the KEGG database (http://www.genome.jp/kegg/) and the PseudoCyc database (www.pseudomonas.com:1555/).

malate, fumarate and succinate as carbon and energy sources, and the transport system for C_4 dicarboxylates has recently been described (Rojo, 2010; Valentini *et al.*, 2011). An upregulation of the C_4-dicarboxylate transport system in two persistent isolates compared to two intermittent colonizing isolates has previously been shown by transcriptomic analysis (Tramper-Stranders *et al.*, 2012), a finding that led to the hypothesis that the C_4-dicarboxylates transport system may be important for early persisting infection with *P. aeruginosa*. However, some of the *P. aeruginosa* isolates included in the present study showed reduced respiration on succinate compared to the controls (Tables 2 and 3), suggesting that upregulation of the C_4-transport system is not absolutely required for persistence in the CF lung.

Mutations in global regulators and metabolic phenotypes

We have compared the catabolic profiles of our laboratory mutants and the clinical isolates harbouring mutations in the same genes, to attempt to attribute a given phenotype to a global regulator mutation. However, as the clinical isolates may have several other mutations besides *mucA*, *lasR* and *rpoN*, this comparison is tentative.

rpoN has been shown to play an important role in the regulation of catabolic pathways in *P. aeruginosa* (Nishijyo *et al.*, 2001) as the structural gene for glutamine synthetase, which catalyses the conversion of L-Glu to L-Gln (a precursor for several amino acids, purines and pyrimidines) is under RpoN control (Totten *et al.*, 1990) and by being

Table 6. The carbon and nitrogen sources showing respiratory reduction for M compared to NM isolates

Intermittent		Chronic					
CF405	**CF408**	**CF312**		**CF341**	**CF483**	**CF519**	**CF544**
Carbon		**Carbon**					
No change	No change	L-Ala	α-Ketobutyrate	L-Ala	L-Asp	No change	No change
		L-Arg	β-Hydroxybutyrate	L-Asn	L-Glu		
		L-Asn	DL-Citramalate	L-Asp	*N*-Acetyl-L-Glu		
		L-Ile	DL-Malate	L-Glu	Succinate		
		L-Leu	Malonate	L-Ile	Inosine		
		L-Pro	Propionate	L-Pro			
		N-Acetyl-L-Glu	Quinate	Tyramine			
		Tyramine	D-Arabitol	Pyruvate			
		Pyruvate	D-Mannitol	D-Fructose			
		Citrate	D-Ribose	D-Gluconate			
		Fumarate	Glycerol	D-Mannitol			
		L-Malate	Inosine	δ-Aminovalerate			
		Acetate					
Nitrogen		**Nitrogen**					
No change	No change	L-Arg	Putrescine	Agmatine	No change	No change	No change
		L-Pro	Tyramine	Tyramine			
		L-Ornithine	Guanine				
		Histamine					

involved in the two-component regulatory system CbrA–CbrB, *rpoN* controls the utilization of multiple carbon and nitrogen sources in *P. aeruginosa*. In accordance, the *rpoN* mutant catabolized L-Arg and L-His poorly, a phenotype previously described for *cbrA–cbrB* mutants (Nishijyo *et al.*, 2001).

Interestingly, despite the respiratory reduction on many carbon and nitrogen sources, the mucoid *rpoN* mutants of CF312 survived in the lung of the patient for the next 10 years as observed by sequencing the later isolates (data not shown). New phenotypic profiles with the supply of different carbon sources, which allow better respiration of the *rpoN* mutants, such as Gln on which respiration is seen (Totten *et al.*, 1990), may more accurately determine the metabolic capabilities of these isolates.

Conversion to the M phenotype is a hallmark of chronic *P. aeruginosa* CF lung infection, as it represents biofilms in the CF airways (Frederiksen *et al.*, 1997; Pressler *et al.*, 2006). The finding of M isolates in intermittently colonized patients in our study confirms that with early and intensive treatment, alginate-hyperproducer strains can also sometimes be eradicated (Döring *et al.*, 2012). To determine if the catabolic profiles of the M CF isolates could be explained by their *mucA* mutations, we compared them to a *mucA* mutant laboratory strain PDO300 (ΔG430). Seven of the eight carbon sources showing respiratory reduction for PDO300 also showed respiratory reduction for some clinical M isolates (Table S12).

The laboratory *lasR* mutant tested here had respiratory improvement on three carbon sources (Table S11). This represents fewer changes compared to a previous study (D'Argenio *et al.*, 2007), which can be explained by the different background of the mutants (PAO1 and PA14, respectively). A study has shown that *lasR* mutant *P. aeruginosa* becomes resistant towards the antibiotics tobramycin and ciprofloxacin (Hoffman *et al.*, 2010). Sensitivity towards these antibiotics is, however, not impaired in the *lasR* mutant CF isolates or in our laboratory mutant. Most of the CF isolates tested retained their protease

Table 7. Carbon and nitrogen sources showing respiratory changes in the lung (r)/sinus (t) isolate pair comparison

Intermittent					Chronic
CF405*	**CF408†**	**CF414***	**CF430†**	**CF431***	**CF341†**
		Carbon			**Carbon**
L-His	α-D-Glucose	L-Ala	L-Ala	L-Asp	L-Ala
L-Leu	D-Fructose	L-Asp	L-Asp	L-Glu	L-Asp
D-Ala	D-Mannitol	L-Glu	L-Ile	L-Ornithine	L-Glu
δ-Aminovalerate	γ-Aminobutyrate	L-Pro	L-Pro	Tyramine	L-Ile
β-Hydroxybutyrate	δ-Aminovalerate	*N*-Acetyl-L-Glu	D-Ala	D-Arabitol	L-Pro
D-Ribose		4-Hydroxybenzoate	L-Malate	Inosine	Succinate
Inosine		D-Gluconate	γ-Aminobutyrate		Acetate
Pyruvate		D-Mannitol	*N*-Acetyl-L-Glu		D-Arabitol
		Glycerol	α-D-Glucose		D-Fructose
		L-Lactate	D-Fructose		Inosine
		p-Hydroxy-phenylacetate	D-Gluconate		
			D-Mannitol		
		Propanoate	Glycerol		
			β-Hydroxybutyrate		
			4-Hydroxybenzoate		
		Nitrogen			**Nitrogen**
No change	No change	L-Trp	L-Met	D-Ser	Agmatine
		Thymine	L-Trp	Nitrate	
		Ammonia	L-Tyr		
		Nitrate	L-Citrulline		
		Nitrite	Formamide		
		Urea	Nitrite		

*Respiratory reduction.
†Respiratory improvement.
(r) reference strain
(t) test strain

activity, though a decrease between the early and late isolates was generally observed.

In regard to the development of auxotrophy in *P. aeruginosa* CF isolates as shown before (Barth & Pitt, 1995, 1996; Thomas *et al.*, 2000), we do not attribute this as the likely explanation for the observed metabolic changes on amino acid substrates, as the method applied tests the ability of the isolates to take up the given substrate and incorporate it into the right pathways, and therefore only indirectly the ability to synthesize it.

Due to the versatility of the metabolic system of *P. aeruginosa*, it is difficult to know whether the affected metabolic pathways actually are compromised and result in metabolically impaired bacteria, or if the bacteria instead react by circumventing the described pathways.

Our *in vitro* experiments do not necessarily reflect the *in vivo* situation in the CF airways, but rather the ability of the bacteria to display a set of chosen properties in *in vitro* standardized conditions. Another limitation of our study is that it has been conducted on single sputum isolates. These might be representative for the bacterial population in the intermittently colonized patients, but have the risk of being less representative for the chronically colonized patients, where a higher heterogeneity of phenotypes has been observed. Applying PMs on several colonies from subsequent sputum samples would answer the question of the dynamic of catabolic heterogeneity of the isolates in the sputum bacterial population. Mapping of the substrates selected for analysis to the given pathways indicates how parts of the pathway function, and does not tell us the overall functionality of the pathway. We can therefore only estimate the metabolic consequences for the isolates of having either reduced or improved respiration on the particular substrates, especially for the larger pathways.

It has been shown that *P. aeruginosa* grows in both aerobic and anaerobic niches of the CF airways (Schobert & Jahn, 2010). As we have created metabolic profiles of the clinical CF isolates under only aerobic conditions, our data may only partially reflect the actual metabolic capabilities of the isolates. It is possible to perform PM analysis under anaerobic conditions, and these data may give a more accurate picture of the metabolism of the CF *P. aeruginosa* isolates.

In conclusion, our findings using PMs on a relatively limited number of isolates show extensive heterogeneity of the catabolic profiles of *P. aeruginosa* isolates from CF patients, as a result of the heterogeneous CF lung environment. Our hypothesis that major metabolic changes occur in the clinical *P. aeruginosa* isolates during the early adaptation phase was difficult to test on the chosen material. The early metabolic phenotypic changes may represent stochastic gain and loss mutations, where the *mucA* mutation offers adaptive fitness to the CF lung by promoting biofilm growth, and thereby protection against the immune response of the host and antibiotic therapy (Bjarnsholt *et al.*, 2009; Høiby *et al.*, 2010a).

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