Pseudomonas aeruginosa displays an altered phenotype *in vitro* when grown in the presence of mannitol

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ABSTRACT

D-mannitol has been approved in dry powder formulation as an effective antimucolytic agent in patients with cystic fibrosis. What is not known is the effect of adding a metabolisable sugar on the biology of chronic bacterial pathogens in the CF lung. Therefore, a series of simple *in vitro* experiments were performed to examine the effect of adding D-mannitol on the phenotype of the CF respiratory pathogens *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. Clinical isolates (n=86) consisting of *P. aeruginosa* (n=51), *B. cenocepacia* (n=26), *P. putida* (n=4), *Stenotrophomonas maltophila* (n=3) and *Pseudomonas* spp. (n=2) were examined by supplementing basal nutrient agar with varying concentrations of D-mannitol (0–20% [w/v]) and subsequently examining for any change in microbial phenotype. The effect of supplementation with mannitol was four-fold, namely i) To increase the proliferation and increase in cell density of all CF organisms examined, with an optimal concentration of 2–4% (w/v) D-mannitol. No such increase in cell proliferation was observed when mannitol was substituted with sodium chloride. ii) Enhanced pigment production was observed in 2/51 (3.9%) of the *P. aeruginosa* isolates examined, in one of the *P. putida* isolates, and in 3/26 (11.5%) of the *B. cenocepacia* isolates examined. iii). When examined at 4.0% (w/v) supplementation with mannitol, 11/51 (21.6%) *P. aeruginosa* isolates and 3/26 (11.5%) *B. cenocepacia* isolates were seen to exhibit the altered adhesion phenotype. iv). With respect to the altered mucoid phenotype, 5/51 (9.8%) *P. aeruginosa* produced this phenotype when grown at 4% mannitol. Mucoid production was greatest at 4%, was poor at 10% and absent at 20% (w/v) mannitol. The altered mucoid phenotype was not observed in the *B. cenocepacia* isolates or any of the other clinical taxa examined. Due consideration therefore needs to be given, where there is altered physiology within the small airways, leading to a potentially altered biological state of the colonising microorganisms in novel inhaled pharmaceutical interventions in CF, particularly those, which are not designated as antimicrobial agents.

KEY WORDS: Alginic acid.
Mannitol.
Mucus.
Pigmentation.
Pseudomonas aeruginosa.
Saline solution, hypertonic.

Introduction

Outside of pulmonary exacerbation, the relative microbiological stability of the cystic fibrosis (CF) lung is a fine equilibrium of multiple factors. In microbiological terms, these include the presence/absence of bacterial pathogens, numbers of organisms present, antibiotic resistance, carriage/expression of bacterial virulence determinants, relative phase of bacterial growth (e.g., presence of senescent cells/persisters), host/environmental stress responses and relative nutritional/starvation status of bacterial pathogens, iron sequestration and competition and the dynamic flux from the co-habiting microbiome of the lung. Several other host (patient)-related factors can also contribute to this instability in this two-host (patient and bacterium) system.

In microbiological terms, what is not that well understood is the sequencing and interactions of these factors, when they occur together in a particular pattern, what precipitates the tipping of the relatively stable CF lung into a pulmonary exacerbation. In addition, the relative contribution and interaction of the human host undoubtedly plays a significant part in driving this stable equilibrium to a state of relative instability and hence onward to a pulmonary exacerbation.

The 21st century is witnessing the development of many new pharmacological interventions in CF, in order to ameliorate the effects of cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction and thus improve patient outcomes. One such intervention is the use of dry powder mannitol, as an effective antimucolytic agent in the lung.[1] Inhaled mannitol also promotes effective coughing and stimulates mucociliary clearance. The beneficial effect of mannitol on mucus and its clearance has been demonstrated in patients with asthma, bronchiectasis and cystic fibrosis.[1] While this intervention is not designed as anti-infective, the effect (if any) of adding D-mannitol, a hexahydric alcohol and metabolisable energy source, to the high density of bacterial pathogens (~10^8–10^9 colony

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Table 1. Effect of mannitol supplementation (0–20% [w/v]) on growth dynamics of *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Stenotrophomonas maltophilia*.

	Concentration of mannitol (%w/v)					
Organism	0%*	0.5%	2.0%	4.0%	10%	20%
Pseudomonas aeruginosa (*n*=30)	+	++	+++	+++	++	+/–
Burkholderia cenocepacia (*n*=26)	+	++	+++	+++	++	+/–
Stenotrophomonas maltophilia (*n*=3)	+	++	+++	+++	++	+/–

*Unsupplemented nutrient agar.

All isolates grew with 20%(w/v) mannitol.

No growth enhancement with NaCl supplementation and no growth >4.5% (w/v) NaCl

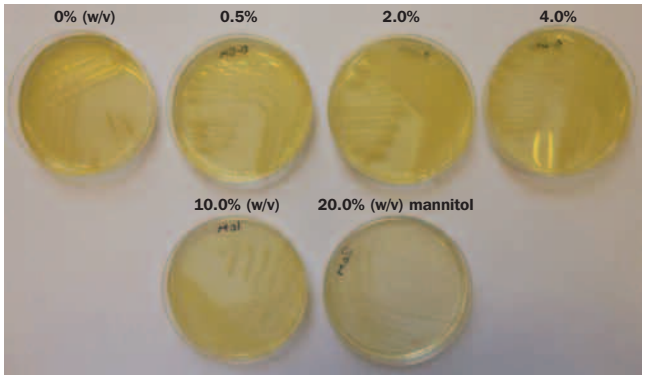

Fig. 1. Effect of mannitol supplementation (0–20% [w/v]) on growth dynamics of *Pseudomonasa aeruginosa*, *Burkholderia cenocepacia* and *Stenotrophomonas maltophilia*.

forming units(cfu)/g sputum[2,3] within the CF lung is unknown. It therefore could be reasonably hypothesised that alteration of this finely tuned physiological milieu may upset this equilibrium and drive this to relative instability, due to utilisation of this 6-carbon molecule and a change of growth status within the CF airways.

Therefore, it is the aim of this study to examine the *in vitro* effect on the bacterial phenotype of several common Gram-negative bacterial pathogens by supplementation of microbiological basal agar with varying concentrations of D-mannitol.

Materials and methods

Bacterial isolates

Clinical isolates (*n*=86), consisting of *Pseudomonas aeruginosa* (*n*=51), *Burkholderia cenocepacia* (*n*=26), *P. putida* (*n*=4), *Stenotrophomonas maltophila* (*n*=3) and *Pseudomonas* spp. (*n*=2) were obtained from the Northern Ireland Health and Social Care Microbiology Repository, (MicroARK; www.microark.com). All the isolates had been grown previously from the sputum of adult CF patients and had been archived at –80°C. All isolates were recovered on Columbia blood agar (Oxoid CM0031, Oxoid , Basingstoke, UK), supplemented with 5% (v/v) defibrinated horse blood for 24 h at 37°C, under aerobic conditions and passaged a further three times prior to use. In selecting the *P. aeruginosa* isolates for this study, all isolates were prescreened for 'mucoidy' and any organism displaying this was excluded.

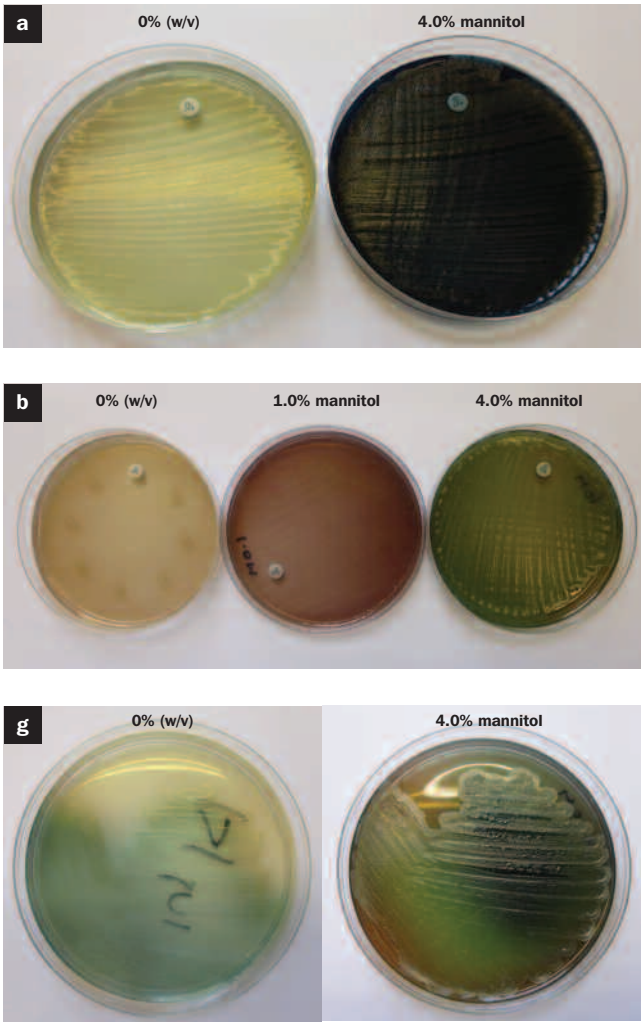

Fig. 2. Effect of mannitol supplementation at **a)** 0%, **b)** 1% and **c)** 4% on pigment production of *Pseudomonasa aeruginosa*.

Altered phenotype studies

P. aeruginosa (*n*=51), *B. cenocepacia* (*n*=26) and *S. maltophila* (*n*=3) were examined in this experiment. Bacterial growth in the presence of five concentrations of mannitol were examined, whereby basal nutrient agar (NA) plates (Oxoid CM1) were supplemented with D-mannitol (Sigma, USA) to give final mannitol concentrations of 0.5% (w/v), 2.0%, 4.0%, 10% and 20% mannitol concentrations. Unsupplemented NA plates without the addition of mannitol (0%) served as

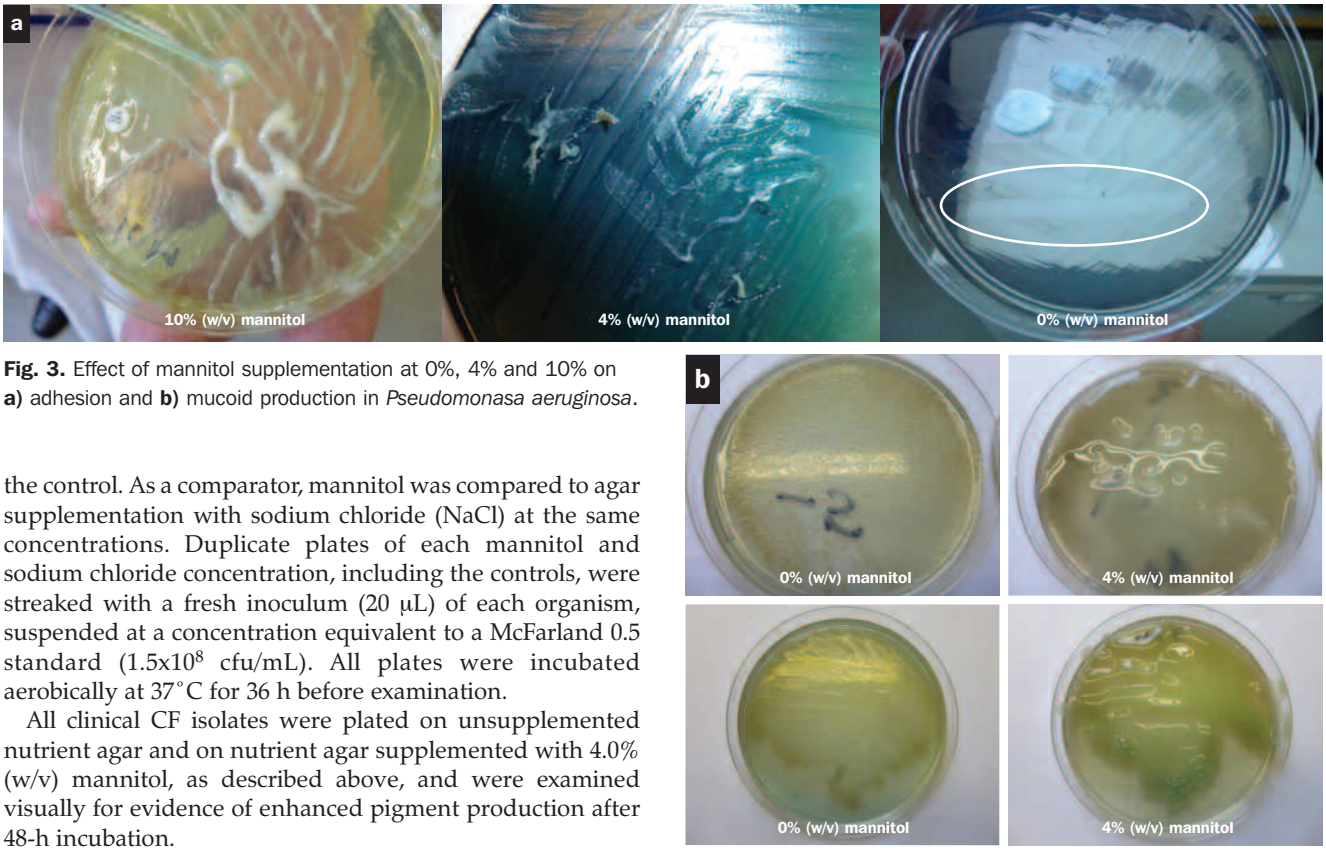

Fig. 3. Effect of mannitol supplementation at 0%, 4% and 10% on **a)** adhesion and **b)** mucoid production in *Pseudomonasa aeruginosa*.

the control. As a comparator, mannitol was compared to agar supplementation with sodium chloride (NaCl) at the same concentrations. Duplicate plates of each mannitol and sodium chloride concentration, including the controls, were streaked with a fresh inoculum (20 µL) of each organism, suspended at a concentration equivalent to a McFarland 0.5 standard ($1.5x10^8$ cfu/mL). All plates were incubated aerobically at 37˚C for 36 h before examination.

All clinical CF isolates were plated on unsupplemented nutrient agar and on nutrient agar supplemented with 4.0% (w/v) mannitol, as described above, and were examined visually for evidence of enhanced pigment production after 48-h incubation.

All clinical CF isolates were plated on unsupplemented nutrient agar and on nutrient agar supplemented with 4.0% (w/v) mannitol, as described above, and were examined after 48-h incubation for evidence of i) an adhesion phenotype and ii) a mucoid phenotype. The adhesion phenotype was defined at examination, where the organism had an altered moist and 'sticky' phenotype in comparison with the unsupplemented control, which was stringy on examination with an inoculation loop, but which fell short of 'mucoidy'. The mucoid phenotype was defined at visual examination, where the organism produced large amounts of extracellular polysaccharide material. In addition, the altered mucoid phenotype could be reversed to the wild-type non-mucoid phenotype on subsequent re-subculture on nutrient agar.

Results

Enhanced colony growth and proliferation

The effect of supplementation with mannitol and sodium chloride is shown in Table 1 and Figure 1.

Enhanced pigment production

Enhanced pigment production was observed in 2/51 (3.9%) of the *P. aeruginosa* isolates examined, in one (1/4 isolates) of the *P. putida* isolates and in 3/26 (11.5%) of the *B. cenocepacia* isolates examined. Illustration of this enhanced pigment production is shown in Figures 2a–c.

Enhanced adhesin phenotype and mucoid production

When examined at 4.0% (w/v) supplementation with mannitol, 11/51 (21.6%) *P. aeruginosa* isolates and 3/26 (11.5%) *B. cenocepacia* isolates were seen to exhibit the altered adhesion phenotype. With respect to the altered mucopid phenotype, 5/51 (9.8%) *P. aeruginosa* produced this phenotype when grown at 4% mannitol. Mucoid production was greatest at 4%, was poor at 10% and absent at 20% (w/v) mannitol. The altered mucoid phenotype was not observed in the *B. cenocepacia* isolates or any of the other clinical taxa examined. Pictorial illustration of the altered adhesion phenotype and the altered mucoid phenotype is seen in Figures 3a and 3b, respectively.

Discussion

Historically, it has been known that *P. aeruginosa* and *B. cenocepacia* can utilise mannitol, as a sole carbon source for growth.[4] This text also cites mannitol utilisation as a differentiating test to aid in the characterisation of the different species within the genus *Pseudomonas*. More recently in the genomics era, we can now identify the genes responsible for mannitol utilisation by Gram-negative CF bacterial pathogens; for example, the presence of the mannitol dehydrogenase gene at position 2586434 – 2587909 in *P. aeruginosa* PAO1 (GenBank Accession AE004091.2). Furthermore, we know that the CF lung harbours large numbers of bacterial organisms in both stable[2] and pulmonary exacerbation states,[3] which are in the order of 10^7–10^8 cfu/g sputum.

Relatively little is known about the nutritional status of CF bacterial pathogens that are chronically colonising the CF lung. Survival of organisms in the CF lung is extremely challenging from a microbiological perspective, due to the competitive effects of the co-habiting microflora. We now know from microbiome studies that the lung has a highly complex taxonomical structure of several hundred taxa,[5]

with as yet undefined inter- and intra-taxa mechanisms of ecological survival/succession. The complexity of this taxonomical structure and numbers of bacteria will have specific nutritional requirements, which may become exhausted in the absence of a sustainable source of essential nutrients and an energy source. Therefore, the sudden alteration of this milieu through the addition of D-mannitol presents new opportunities for the co-habiting organisms.

In these relatively simple bacteriological studies, we have attempted to gain a better understanding of how the phenotype of the major Gram-negative pathogens *P. aeruginosa* and *B. cenocepacia* react *in vitro* to varying concentrations of mannitol. Mannitol concentrations ranging from 0% to 20% were selected for investigation, as we are not aware of any reports detailing the real-life concentratiuon of mannitol in epithelial surface liquid or in sputum. It is to be expected that dosing of the CF lung with dry powder mannitol will not result in a homogenous concentration of mannitol throughout the treated small airways, but rather a heterogenous combination of concentrations and indeed microniche concentrations that could reflect a wide range of concentrations, as reflected in the concentration range selected for these investigations. Any resulting altered phenotype could be the result of several effects, including i) availability of a new and utilisable carbon source, ii) availability of a new energy source for bacterial metabolism, and iii) stress response on the colonising bacteria in the airways due to the acute presence of an osmotic agent. Presently, we do not have any available data detailing concentration of mannitol in the epithelium surface liquid within the small airways and alteration to the water availability (Aw) factor, which could drive increased stress responses and potential ecological shifts due to varying Aw.

The universal increase in cell proliferation in *P. aeruginosa* in these studies is not surprising, given the well-established biochemical pathways of mannitol catabolism in this organism.[6] What was slightly surprising was this relative growth burst, even in the presence of large amounts of available carbon, nitrogen and energy which were present in the relatively nutrient-rich nutrient agar basal medium employed throughout this series of experiments. Following on, these experiments showed an altered phenotype exhibiting an adhesion type and a mucoid type in approximately 22% and 10% of *P. aeruginosa* isolates, respectively. The biochemical pathways of mannitol to alginate in *P. aeruginosa* has been well characterised previously.[7] Exogenous mannitol is converted initially to fructose-6-phospahte (F6P) by mannitol dehydrogenase, and its entry into the bacterial cell is initially aided by the mannitol active transport system. F6P then proceeds to alginate, as detailed in the pathway shown in Figure 1. From our studies, only approximately 10% of clinical isolates were able to produce alginate by supplementation with mannitol.

Finally, our studies demonstrated increased pigment production in approximately 4% of *P. aeruginosa* isolates examined (Figures 2a–c). Pigment production in *P. aeruginosa* involves the redox-active pigment pyocyanin. Pyocyanin promotes virulence by interfering with several cellular functions in host cells including electron transport, cellular respiration, energy metabolism, gene expression and innate immune mechanisms.[8]

All of the data gathered to date is exclusive to the *in vitro* state. As yet, we do not have any data to indicate that any of these altered phenotypes exist in the *in vivo* state within the host's CF airways. Given the potential for increased virulence of *P. aeruginosa* exhibiting these four altered phenotypes *in vivo*, urgent studies are required to show what happens to the bacterial phenotype *in vivo*.

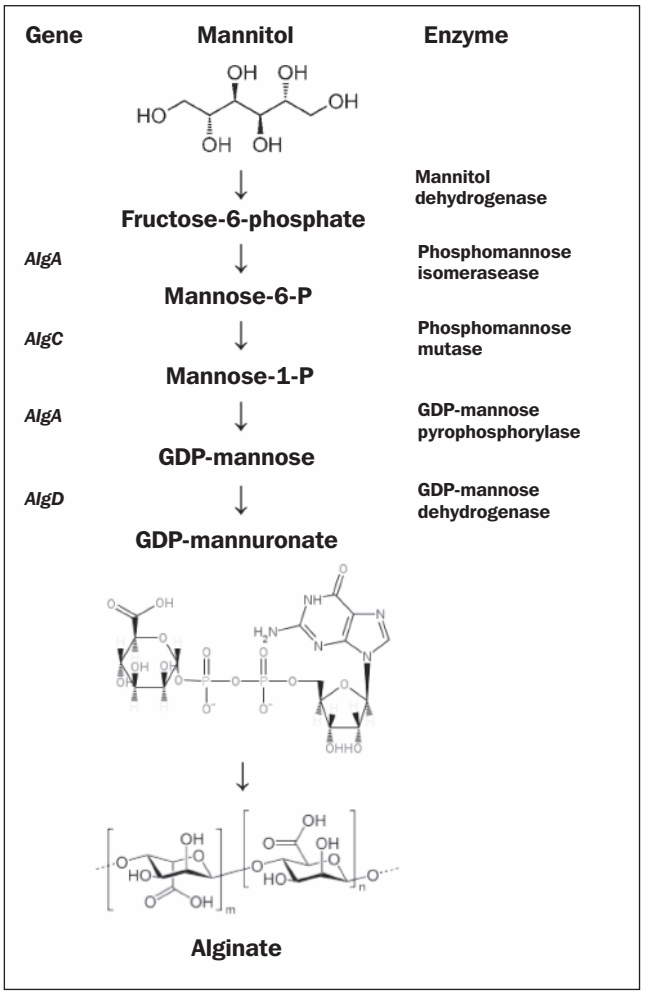

Fig. 4. Biosynthetic pathway of mannitol to alginate in *Pseudomonas aeruginosa*.

In terms of methodical implications of our findings, all of this work was performed on mannitol-supplemented and -unsupplemented basal agar. Given that most CF clinical microbiology laboratories would not be performing diagnostic tests on mannitol-supplemented media, such phenotypic variants would probably go unnoticed. The consequences of this would possibly be that the laboratory reports the 'laboratory phenotype' (i.e., the observation of growth characteristics as defined by the artificial cultural conditions *in vitro*, which may not be reflective of the *in vivo* phenotype in the lungs of the CF patient). Such a phenomenon is not unique to mannitol, but exists with all other inhaled therapies, where there is a disconnect between diagnostic laboratory conditions and the emulation of the *in vivo* physiological milieu in the CF lung.

Similar work with mannitol and *Burkholderia* organisms has demonstrated that when *B. multivorans* is grown on a mannitol-rich medium, there is a genome-wide transcriptional response that impacts on multiple virulence traits in an exopolysaccharide-independent manner.[9] In

addition, other work with the same species demonstrates an upregulation of newly identified fimbrial and afimbrial adhesions.[10] Reid and Bell[11] recently raised clinical concerns regarding potential polysaccharide production in *B. cepacia* complex (BCC) organisms isolated from CF patients, as such patients with one member of this BCC complex, namely *B. cenocepacia*, were excluded from clinical trials involving mannitol, and these authors have advised scrutiny in such patients taking inhaled mannitol therapy.

More recently, workers in Australia have demonstrated a further alteration to the *P. aeruginosa* phenotype when grown in the presence of mannitol (10–40 mmol/L). In this case, the presence of mannitol increased tobramycin sensitivity of persister cells up to 1000-fold, through an active physiological response.[12]

In conclusion, this study showed that *in vitro* supplementation of basal growth media with D-mannitol elicited four altered phenotypes to varying degrees in clinical isolates of *P. aeruginosa*, including: i) enhanced cell growth/cell density, ii) enhanced pigment production, iii) expression of adhesins, and iv) expression of 'mucoidy'.

Due consideration therefore needs to be given where there is altered physiology within the small airways, leading to a potentially altered biological state of the colonising microorganisms in novel inhaled pharmaceutical interventions in CF, particularly those not designated as antimicrobial agents. Further studies are now warranted to establish if such altered phenotypes exist in bacterial pathogens in the CF airways *in vivo*, with patients on mannitol dry powder inhalation, and, if so, do such altered phenotypes affect clinical outcomes? □

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