BACTERIAL PATHOGENICITY

The high amino-acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic *Pseudomonas aeruginosa*

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Many isolates of *Pseudomonas aeruginosa* from cystic fibrosis (CF) patients are auxotrophic and require amino acids for growth. A quantitative assay was used to determine the total content of free amino acids of sputum sol-phase extracts from CF and non-CF patients to assess the presence of amino acids in the airway. CF patients colonised with auxotrophic *P. aeruginosa* had a higher sputum amino-acid content (mean 6.77 mg/ml) than those colonised with prototrophs (mean 3.77 mg/ml); overall, CF specimens (mean 5.70 mg/ml) had a higher amino-acid content than non-CF samples (2.52 mg/ml). The amino-acid profile of sputum extracts was assessed by one-dimensional thin layer chromatography (TLC). Several amino acids were identified in the extracts, in particular, leucine, isoleucine, phenylalanine, tyrosine, alanine, serine and methionine or valine or both. All sputum specimens except two (which contained <1.5 mg of amino acids/ml), promoted the growth, of 34 auxotrophic strains of *P. aeruginosa* from CF patients in a minimal medium. These results indicate, therefore, that amino acids are plentiful in the sputum of CF patients and are able to supply the requirements of auxotrophic strains. It is suggested that the increased amino-acid content in the airways of CF patients plays a significant role in the selection and maintenance of nutritionally deficient *P. aeruginosa*.

Introduction

Deterioration of lung function in patients with cystic fibrosis (CF) is one of the major determinants of the high morbidity and mortality associated with the disease [1]. The airways of CF patients are invariably colonised by *Pseudomonas aeruginosa* which, during the course of the disease, undergoes many phenotypic variations. Production of alginate exopolysaccharide [2], sensitivity to human serum [3], hypersensitivity to semi-synthetic penicillins [4] and other phenotypic alterations appear to be a result of the adaptation of the bacterium to the environment in the CF lung. This adaptability extends to the nutritional status of the organism with many strains of *P. aeruginosa* from CF exhibiting auxotrophy for amino acids [5]. These variants are derived, in the lung, from prototrophic nutritionally efficient wild-type strains [6].

The physical and chemical properties of the CF

airways secretions are also altered in comparison with normal subjects. In an early study on the composition of mucus from pulmonary secretions, Potter *et al.* [7] showed a wide spectrum of values for DNA, protein, lipid and carbohydrate in 'normal' (laryngectomised patients), bronchiectatic, and CF patients; the highest values were found in the CF group. Later, Lopez-Vidreiro and Reid [8] concluded that the quantitative changes in mucus composition were secondary to infection rather than a primary abnormality of the disease. Abnormalities were also described in the ionic composition of CF sputum by Kilbourn [9] who correlated an increased calcium concentration with colonisation by *Staphylococcus aureus*, and an increased magnesium content with the presence of mucoid *P. aeruginosa* [10].

Given the presence of amino acid-requiring *P. aeruginosa* variants in CF, it is necessary to show that sputum provides these compound for strains to utilise. Ohman and Chakrabarty [11] studied sputum from two CF patients and suggested that free amino acids were increased in comparison with controls, but they were primarily concerned with the effect of those compounds on alginate production by *P. aeruginosa*.

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In this study, the amino-acid content and profile of CF sputa were first determined and then the results were compared with those of non-CF specimens.

Materials and methods

Bacterial isolates

Thirty-four *P. aeruginosa* isolates were obtained from CF patients and confirmed as auxotrophs for their nutritional status as described previously [6]. *P. aeruginosa* NCTC50184 (met⁻) auxotrophic for methionine was used as a control strain, as well as a prototrophic isolate from a CF patient.

Sputum collection and processing

Sputum samples from 26 non-CF patients were obtained from the Microbiology Laboratory of The Royal Brompton Hospital, London and from the Department of Microbiology of the Watford General Hospital, London. The information provided on the request form with the sputum was used as clinical data from the non-CF patients. Sputum from 28 CF patients were obtained as described previously [6].

Sputum samples were homogenised by shaking with an equal volume of Ringer's solution and glass beads for c. 3 min. The homogenates were autoclaved at 121°C for 15 min and centrifuged at 11 600 *g* for 30 min. The supernate (sol phase) was removed and stored at -20°C. Sol-phase samples from four patients were subjected to amino-acid determinations before and after autoclaving. Sputum medium was prepared by diluting autoclaved sol-phase sputum extracts 1 in 200 in minimal agar medium (MAM) agar that contained mineral salts and glucose only [12]. The sputum medium plates were inoculated with bacterial suspensions as above and incubated at 37°C for 48 h. Plates of King's A agar (KA) [13], MAM without sputum extracts and MAM containing 20 'standard' [14] amino acids plus hydroxyproline and ornithine, each 20 µg/ml, were also inoculated as above.

Quantitative determination of amino acids

All reagents for the determination of amino acids were purchased from Sigma unless otherwise stated. Sol-phase sputum was assayed for total amino-acid content according to the method of Spies [15] modified by Moore [16]. Briefly, samples were diluted as appropriate and 50 µl were mixed with ninhydrin reagent in 4 × 0.5-inch tubes with metal caps and placed in a boiling water bath. After 20 min the tubes were removed from the bath, 0.5 ml of diluent (equal volumes of *n*-propanol and distilled water) was added and left at room temperature for 15 min. The OD₅₇₀ was read and the amino-acid concentration was calculated with reference to a standard curve for L-leucine.

Thin layer chromatography (TLC) for amino acids

Volumes of 2 µl of sputum sol phase were applied in a 1.5-cm line, 1.5-cm from the bottom of a 10 × 20-cm cellulose-coated aluminium plate (Merck, Darmstadt, Germany). The solvent used was: *N*-butanol 35 ml, acetone (BDH, Poole) 35 ml, glacial acetic acid (BDH) 10 ml and distilled water 10 ml. The chromatogram was developed twice in the same dimension with the solvent front reaching 7.5 cm from the origin. Bands corresponding to amino acids were located by spraying the plate with ninhydrin (BDH). A standard solution containing 22 amino acids as above, each at 0.1 µg/µl, was used as a control. Alternatively, a plate of 20 × 20 cm was used to allow longer development (15-cm solvent front) to increase resolution between bands. In this case the concentration of the amino-acid standards was as follows: hydroxyproline, proline and cysteine, 2 µg/µl; tyrosine, 0.5 µg/µl; methionine, tryptophan and asparagine, 0.2 µg/µl, and all other amino acids, 0.1 µg/µl.

Statistical methods

The mean values for amino acids in sputum from CF and non-CF patients were compared by the Student's paired and unpaired *t* tests.

Results

Quantitative analysis

The reproducibility of results for total amino-acid content of clinical samples was evaluated. The amino-acid content of 23 sol-phase sputum samples (13 from CF and 10 from non-CF) was determined on two different occasions. The results were not significantly different (*p* = 0.7); as no quantitative variation in amino acids was detected for the same sample tested on different occasions (Table 1). Sputum samples were also compared for the effect of autoclaving on the amino acid content. Four pairs of samples (before and after autoclaving) were compared and this process did not significantly affect the total amino-acid content (*p* = 0.2) (Table 1).

CF sputum data

Eighteen of the 28 CF patients were colonised by either auxotrophs alone or both auxotrophic and prototrophic *P. aeruginosa* (Group I). The remainder were either colonised by prototrophic isolates only or not colonised by *P. aeruginosa* (Group II) (Table 2). In general, patients colonised with auxotrophic *P. aeruginosa* had a higher sputum amino-acid content with a mean of 6.77 mg/ml (range 0.96–14.30) compared with Group II which had a mean value of 3.77 mg/ml (range 1.32–10.66) (*p* = 0.0167). Sixteen (89%) of the patients in Group I had sputum amino-acid levels of >4 mg/ml compared with only two patients (20%)

Table 1. Reproducibility and effect of autoclaving on the quantitative determination of amino acids in sputum samples

CF patient no.	Amino-acid content (mg/ml)		Non-CF patient no.	Amino-acid content (mg/ml)	
	D1	D2		D1	D2
2	0.98	0.94	1	1.26	1.58
4	9.48	9.26	2	1.48	1.48
6	6.48	5.80	4	3.04	2.34
13	7.14	7.96	8	1.02	0.84
15	6.18	5.96	9	1.92	1.52
17	4.20	4.28	10	1.60	1.42
34	15.26	13.40	21	0.82	0.80
37	8.76	9.36	23	0.40	0.38
51	10.70	11.20	25	1.92	2.08
47	6.90	7.32	26	9.06	9.66
39	2.93	2.54	D1 vs D2, p = 0.7*		
48	1.76	1.74			
23	0.69	0.77			
Amino-acid content (mg/ml)					
Sample	before autoclaving		after autoclaving		
A	1.36	p = 0.2*	1.26		
B	3.24		3.04		
C	9.96		9.20		
D	1.60		1.60		

D1, first determination; D2, second determination.

*Not significant (paired *t* test).**Table 2.** Amino-acid content of sputum extracts from CF patients

Patient no.	Nutritional status of <i>P. aeruginosa</i> (amino-acid requirement)	Amino-acid content (mg/ml)
Group I		
2	PRO and AUX (met)	0.96
4	PRO and AUX (ser)	9.37
6	PRO and AUX (met)	6.14
7	PRO and AUX (met)	4.20
9	PRO and AUX (NI)	1.46
13	PRO and AUX (leu)	7.55
15	PRO and AUX (his)	6.07
17	PRO and AUX (arg)	4.24
18	PRO and AUX (arg)	6.12
24	PRO and AUX (NI)	6.40
28	PRO and AUX (met)	8.50
30	PRO and AUX (ile & val)	6.60
34	PRO and AUX (trp)	14.30
37	PRO and AUX (met)	9.06
38	PRO and AUX (met)	6.76
47	AUX (met)	7.11
49	PRO and AUX (met)	6.10
51	PRO and AUX (met)	10.95
Group II		
1	PRO	3.06
3	PRO	3.86
5	PRO	1.32
8	PN	3.30
10	PRO	10.66
29	PN	5.00
32	PN	2.64
39	PRO	2.73
48	PRO	1.75
52	PN	3.44

PRO, prototroph; AUX, auxotroph; NI, not identified; PN, culture negative for *P. aeruginosa*.*Group I, patients colonised by either auxotrophs alone or both auxotrophic and prototrophic *P. aeruginosa*; Group II, patients colonised by prototrophic isolates only, or not colonised by *P. aeruginosa*.

from Group II. However, the CF sputum specimen with the lowest amino-acid content (0.96 mg/ml) originated from a patient who harboured both auxotrophic and prototrophic isolates.

CF versus non-CF specimens

The amino-acid content of sputum sol-phase extracts of the 28 CF patients was compared with values for sputum extracts of 26 non-CF patients given in Table 3. The mean amino-acid content for CF and non-CF specimens was 5.70 mg/ml (range 0.96–14.33) and 2.52 mg/ml (range 0.39–9.36) ($p = 0.0002$) respectively. Twenty-two (79%) of CF specimens contained >3 mg of amino acids/ml compared with only 4 (15%) on non-CF specimens (Fig. 1).

Individual comparisons of amino-acid content of the CF Groups I and II with the values for the 26 non-CF patients were made. Although the amino-acid values of CF Group I were significantly higher than for the non-CF population ($p < 0.0001$) there was no difference between the CF Group II and the non-CF population ($p = 0.1979$) (Table 4).

Qualitative analysis by TLC

To identify the profile of individual amino acids all sputum samples were examined by one-dimensional TLC on a 10 cm × 20 cm plate. Each of the 28 sol-phase extracts from CF patients gave a complex but

relatively constant amino-acid profile that varied only in the intensity of bands which appeared to correlate with the total amino-acid content (Fig. 2). The profiles were similar to those given by non-CF specimens that contained sufficient amino acids detectable by chromatography and they generally corresponded to the 22 amino acids standard, but it was difficult to identify precisely individual amino acids in each profile.

The presence of individual amino acids did not appear to be influenced by autoclaving, as the one-dimensional TLC profiles of four pairs of sputum pre- and post-autoclaving were indistinguishable.

The resolution between amino-acid bands in a sample from a CF patient was increased by the use of a 20 cm × 20 cm plate in one-dimensional TLC. Fig. 3 shows that the longer development for the migration of individual bands could be correlated more precisely with the bands of standard amino acids contained in the control pools. A single band related to each amino acid was visualised in the standards, although some compounds, particularly lysine and arginine, had a more diffuse migration pattern than others. It was also found that for most amino acids a concentration of 0.1 µg/µl (final amount per lane of 2 µg) was sufficient to render bands visible in the chromatogram. The exceptions were hydroxyproline, proline, cysteine, tyrosine, methionine, tryptophan and asparagine, which yielded weaker bands at 0.1 µg/µl. To circumvent this problem, higher concentrations of

Table 3. Amino-acid content and clinical data of non-CF patients

Patient no.	Culture	Clinical information	Amino-acid content (mg/ml)
1	No significant growth	Bronchiectasis	1.42
2	No significant growth	Carcinoma	1.48
3	<i>Haemophilus influenzae</i>	Bronchiectasis	1.48
4	No significant growth	Upper respiratory tract infection	2.69
5	<i>P. aeruginosa</i>	Asthma	1.00
6	No significant growth	Not supplied	0.98
7	<i>Branhamella catarrhalis</i>	Asthma	9.20
8	No significant growth	Not supplied	0.93
9	<i>B. catarrhalis</i>	Heart failure/Chest infection	1.72
10	<i>Pseudomonas</i> sp.	Bronchiectasis	1.51
11	No significant growth	Coronary artery by-pass graft	2.90
12	No significant growth	Carcinoma of the lung	1.66
13	<i>H. influenzae</i>	Not supplied	1.64
14	<i>P. aeruginosa</i> (mucoid)	Bronchiectasis	1.28
15	No significant growth	Not supplied	1.20
16	<i>B. catarrhalis</i>	Asthma	3.60
17	<i>H. influenzae</i>	Not supplied	1.64
18	<i>P. aeruginosa</i>	Bronchiectasis	8.96
19	No significant growth	Post coronary artery by-pass	1.80
20	No significant growth	Bronchiectasis	2.48
21	No significant growth	Right lower lobe pneumonia	0.81
22	<i>Streptococcus pneumoniae</i> + normal flora	Clot retention	1.16
23	No significant growth	Persistent cough sputum	0.39
24	<i>Pseudomonas</i> sp. + normal flora	Cough sputum	2.24
25	<i>H. influenzae</i>	Infective pleurisy	2.00
26	<i>Staphylococcus aureus</i>	Atrial fibrillation/Chest infection	9.36

Fig. 1. Distribution of amino-acid content of CF and non-CF sputum samples; — — —, mean.

Table 4. Summary of amino-acid content of sputum specimens from CF and non-CF populations

Patient group	Number of samples	Mean amino-acid content (mg/ml)	Minimum value	Maximum value
CF Group I*	18	6.77	0.96	14.30
CF Group II*	10	3.78	1.32	10.66
CF (Total)	28	5.70	0.96	14.30
Non-CF	26	2.52	0.39	9.36

*See Table 1.

Associations (unpaired *t* test)

CF Group I vs CF Group II $p = 0.0167$ significant

CF (Total) vs Non-CF $p = 0.0002$ highly significant

CF Group I vs Non-CF $p < 0.0001$ highly significant

CF Group II vs Non-CF $p = 0.1979$ not significant

these compounds were used (see *Materials and methods*).

The purple colour developed by bands after location with ninhydrin spray was similar for all amino acids with the exception of bands formed by the imino acids (hydroxyproline and proline), and asparagine which gave a yellow and brown colour, respectively. Some amino acids may generate spots associated with secondary compounds and this was particularly

evident for cysteine, an amino acid which degrades rapidly into autoxidation products (Fig. 3).

Amino acids such as leucine, isoleucine, phenylalanine, tyrosine, alanine, threonine and serine were readily identified by the unique position of their bands. However, a number of bands in the sputum profile may represent multiple amino acids, as some compounds had the same or very similar rates of migration. This was the case for methionine and

Fig. 2. One-dimensional TLC of sputum extracts from selected CF and non-CF patients and amino-acid standards.

Fig. 3. One-dimensional TLC of a sputum extract from a CF patient and the correlation of bands with those of amino-acid pools. Individual compounds are given in vertical order according to position of bands. The most rapidly migrating spot in the complex of proline/aspartic acid/cysteine is believed to be a degradation product of cysteine.

valine, glycine and hydroxyproline, and the least mobile complex of asparagine-arginine-lysine-ornithine-histidine. Some compounds – namely, tryptophan, proline, glutamic acid, glutamine, cysteine and aspartic acid – were not visible in the profiles generated from sputum extracts. There were also two bands in the sputum profile that did not correlate with any of the amino acid standards: a yellow spot immediately above the band associated with threonine and a dense band just below that of serine. A difference in the intensity of bands formed by individual amino acids in the sputum profiles was observed. Bands related to leucine, isoleucine, alanine and serine were slightly stronger than most of the others and probably indicated a higher concentration of these compounds in the sputum.

Two-dimensional TLC did not markedly improve resolution between amino acids over the one-dimensional technique, as there was a significant margin of variation in the relative mobilities of amino acids in different chromatographic runs (data not shown). Nevertheless, the latter did confirm the basic amino-acid constitution of the profile obtained with the one-dimensional technique.

Sputum extracts as source of nutrients

Sol-phase extracts of sputum from 22 of 28 CF patients, and six from the non-CF patients were each incorporated into MAM and tested as substrates for the growth of a panel of 34 auxotrophs which required single or multiple amino acids for growth (Table 5). All CF sputum specimens supported the growth of each of the strains with the exception of that from patient 24 that failed to promote the growth of a single strain that required serine or glycine. Apart from 10 isolates, which were not tested against their sputum of origin, all grew on MAM supplemented with the sputum from which they were isolated. Strain NCTC 50184, auxotrophic for methionine, also grew

on all CF sputum media as did the prototrophic control from a CF patient.

The growth of isolates on sputum medium was compared with the growth on MAM containing 22 amino acids. The growth on the majority of individual CF sputum extracts was equivalent, although less intense, to the growth on the pool of amino acids (Fig. 4). Growth was poorest on CF sputum extracts with lower amino-acid content. This was particularly evident for the extracts from CF patients 2 and 9, which had very low amino-acid concentrations (<1.5 mg/ml). The latter were variable in their ability to support the growth of auxotrophs; some isolates grew more luxuriantly than others but, overall, most grew poorly in comparison with MAM supplemented with other sputum or amino-acids pool. It was also observed that some isolates produced more mucoid growth on MAM plus amino acids than on KA.

With the exception of the sputum extract from patient 7, which promoted exuberant growth of all the auxotrophs, the other five specimens tested from non-CF patients (nos. 1, 2, 4, 8 and 9) supported only very poor or no growth of the isolates. Here again, the growth of auxotrophs was associated with a high amino-acid content in the samples.

It is noteworthy that of the 34 CF isolates, one grew as mucoid colonies only on KA, two isolates were mucoid on both KA and MAM containing 22 amino acids, and five had the mucoid characteristic evident only on MAM containing amino acids. Four of the latter required methionine for growth.

Discussion

There are conflicting views as to the nutritional status of the sputum of CF patients. Ohman and Chakrabarty [11] showed that two CF patients' sputa had relatively high concentrations of total amino acids and contained a range of nutrients necessary to support the growth and production of large amounts of alginate by *P. aeruginosa*, but Speert *et al.* [17] postulated, without sputum analysis, that *P. aeruginosa* grew under energy-limiting conditions in the CF broncho-pulmonary milieu and it was this starvation of nutrients that led to the mutation to mucoidy and loss of O antigen by strains.

Although rare in other diseases, auxotrophy is a common phenomenon among bacteria colonising CF patients [5, 18, 19]. Earlier studies demonstrated that the auxotrophic variants of *P. aeruginosa* and *Burkholderia cepacia* were derived from the prototrophic wild-type strains [6, 19] but the mechanism of selection of these forms *in vivo* remains obscure. The

Table 5. Amino-acid requirements of auxotrophic *P. aeruginosa* isolates tested for growth on MAM supplemented with sputum extracts

Requirement	Number of isolates (n = 34)
Methionine	18
Isoleucine and valine	3
Leucine	2
Arginine or ornithine	2
Histidine	1
Serine or glycine	1
Isoleucine and valine and leucine	1
Tryptophan	1
Methionine and leucine	1
Aspartic acid or arginine	1
Lysine	1
Lysine and methionine	1
Histidine and methionine	1

Fig. 4. Growth of 34 auxotrophic *P. aeruginosa* isolates from CF and two control strains on: **a**, KA; **b**, MAM; **c**, MAM supplemented with 22 amino acids; **d**, MAM plus sputum extract from patient no. 24 (amino-acid content 6.4 mg/ml); **e**, MAM plus sputum extract from patient no. 34 (14.30 mg/ml); **f**, MAM plus sputum extract from patient no. 9 (1.46 mg/ml).

present study showed unequivocally that CF sputum contains quantitatively more amino acids than non-CF specimens. Indeed, the presence of several individual amino acids was demonstrated by TLC. Furthermore, each of 22 sputum extracts from CF patients with amino-acid content >1.5 mg/ml promoted the growth of virtually all auxotrophic *P. aeruginosa* tested.

The determination of total amino acids with ninhydrin may be subject to chemical interference and therefore absolute values in sputum may vary. However, the aim was to compare the amino-acid content of CF and non-CF specimens and it was reasoned that any interference in the reaction of ninhydrin with NH₂ groups would be similar in both groups of specimens. It was also the case that a precise determination of the presence or absence of some amino acids was not achievable with TLC, but ready access to more sophisticated amino-acid analysis was not available.

The lack of growth of auxotrophic *P. aeruginosa* in sputum extracts, either of CF or non-CF origin, was associated with a lower content of total amino acids, indicating that these compounds need to be present in at least minimal amounts to support the growth of nutritionally deficient *P. aeruginosa*. Furthermore, amino acids may play an important role in cellular growth rate and alginate production by mucoid *P. aeruginosa*, as reduced growth and decrease in alginate yields was correlated with the removal of amino acids and small peptides from sputum [11]. This study showed that some auxotrophic strains were clearly mucoid only in media supplemented with a pool of amino acids.

It is possible that the excess of free amino acids in the lung of CF patients provides conditions in which mutations of strains leading to nutritional deficiency, a putative disadvantage in a natural environment, may not be a significant handicap in comparison with nutritionally sufficient prototrophs. This was first raised by Lwoff (cited in Harder and Kuenen [20]) who, in a study on the establishment of parasitism, suggested that auxotrophic mutants had a selective advantage over prototrophic forms due to energetic economy. The production of certain amino acids by bacteria is an energetically expensive process [21], thus cells that take up these compounds from the external environment may be able to conserve energy. Interestingly, the mutation leading to the non-motile phenotype characteristic of CF *P. aeruginosa* isolates was associated with glutamine auxotrophy and thought to be an adaptive response by the bacterium to conserve energy [22].

Similarly, it is noteworthy that strains bearing the *metB1* mutation, which causes a requirement for methionine in *Escherichia coli*, revert to prototrophy under conditions of methionine starvation at rates 80-fold higher in aged colonies than for growing cultures

[23]. If this can be applied to the situation *in vivo*, one may postulate that the increased free amino acids in the CF airways would be a key factor for the selection and maintenance of auxotrophic forms colonising these patients.

The phenomenon of auxotrophy is relatively uncommon in wild-type bacteria causing infections, but *c.* 1.5% of *E. coli* isolates from urinary tract infection are auxotrophic for cysteine [24] and this is associated with an accumulation of cysteine-homocysteine *in vivo* in patients with decreased renal function [25]. Furthermore, the selection of cysteine-requiring *E. coli* from prototrophic forms *in vitro* was demonstrated to be enhanced by an excess of cysteine and other sulphur compounds [26].

The origin of free amino acids in the sputum of CF patients is not clear. These compounds may be direct exoproducts from the bacterium itself or from its interaction with the host. The release of free amino acid to the external environment by actively growing prototrophic *P. aeruginosa* was not detected. However, preliminary experiments on satellitism between prototrophs and auxotrophs indicate that a minority of prototrophs do produce substances that can support the growth of some nutritionally deficient strains of *P. aeruginosa* (data not shown). The increased levels of amino acids in the CF airways may, therefore, be associated with the action of bacterial exogenous proteases on the human cells or to the inflammatory response of the host, or both. Indeed, Cicmanec and Holder [27] proposed that the role of exoproteases from *P. aeruginosa* is to generate more utilisable nutritional substrates, such as amino acids, that would facilitate the growth of the bacterium to the levels required for systemic invasion.

We propose that *P. aeruginosa* in CF grows in a pool of amino acids and that this selects and perpetuates the state of auxotrophy as a direct response to the amino-acid rich environmental challenge.

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