Supplementary Materials for

Phage therapy with nebulized cocktail BX004-A for chronic *Pseudomonas aeruginosa* infections in cystic fibrosis: a randomized first-in-human trial

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Supplementary data includes:

*Figs. S1 to S10 (in this file)
*Tables S1 to S8 (in this file)
* Supplementary Data 1 to 6 (in attached Excel spreadsheets)
*Source Data (in attached Excel spreadsheet, ordered by figure numbers)

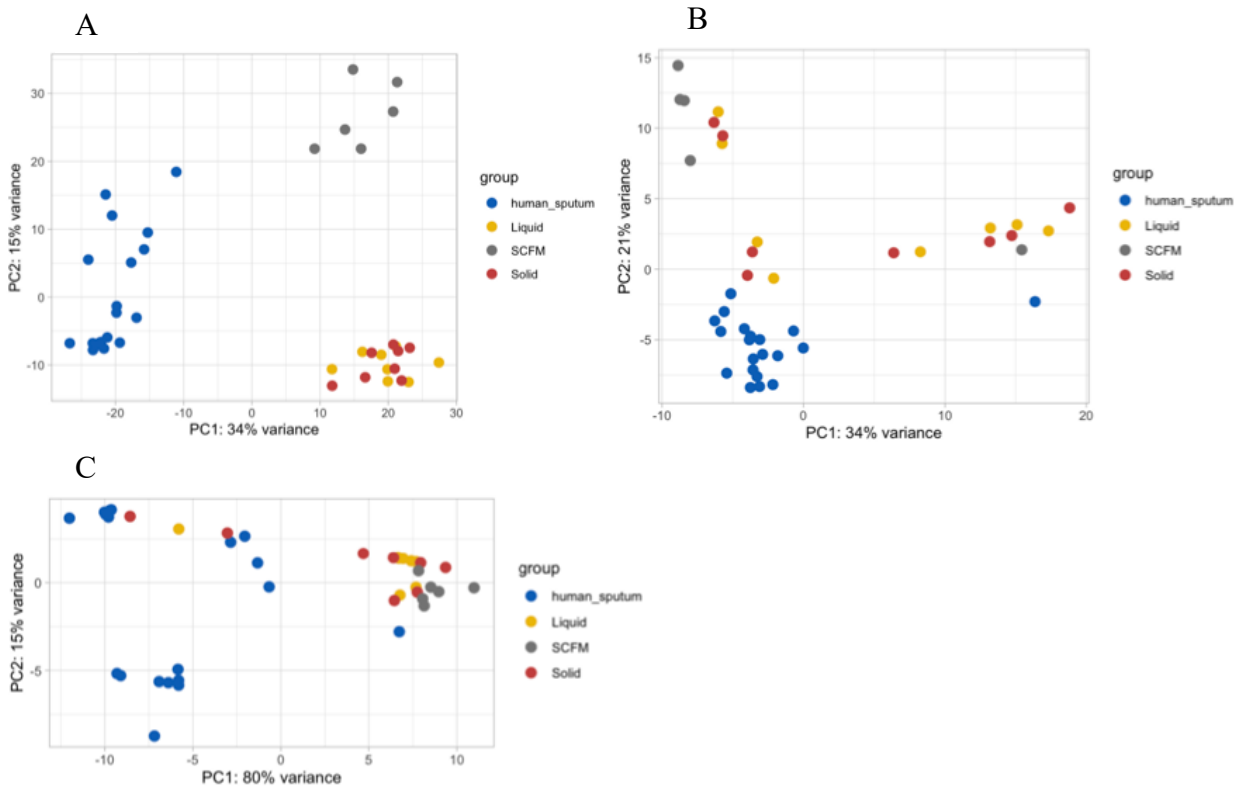

Figure S1. PCA of *P. aeruginosa* transcript relative abundance. (**A**) PCA of all core genes. (**B**) PCA of genes regulating or coding for known phage binding molecules. (**C**) PCA of alginate biosynthesis pathway genes. Sample sizes were: human sputum – 20, SCFM – 6, Solid – 8, Liquid – 8 (see table S4 for further details, technical repeats were averaged). Source data are provided as a Source Data file.

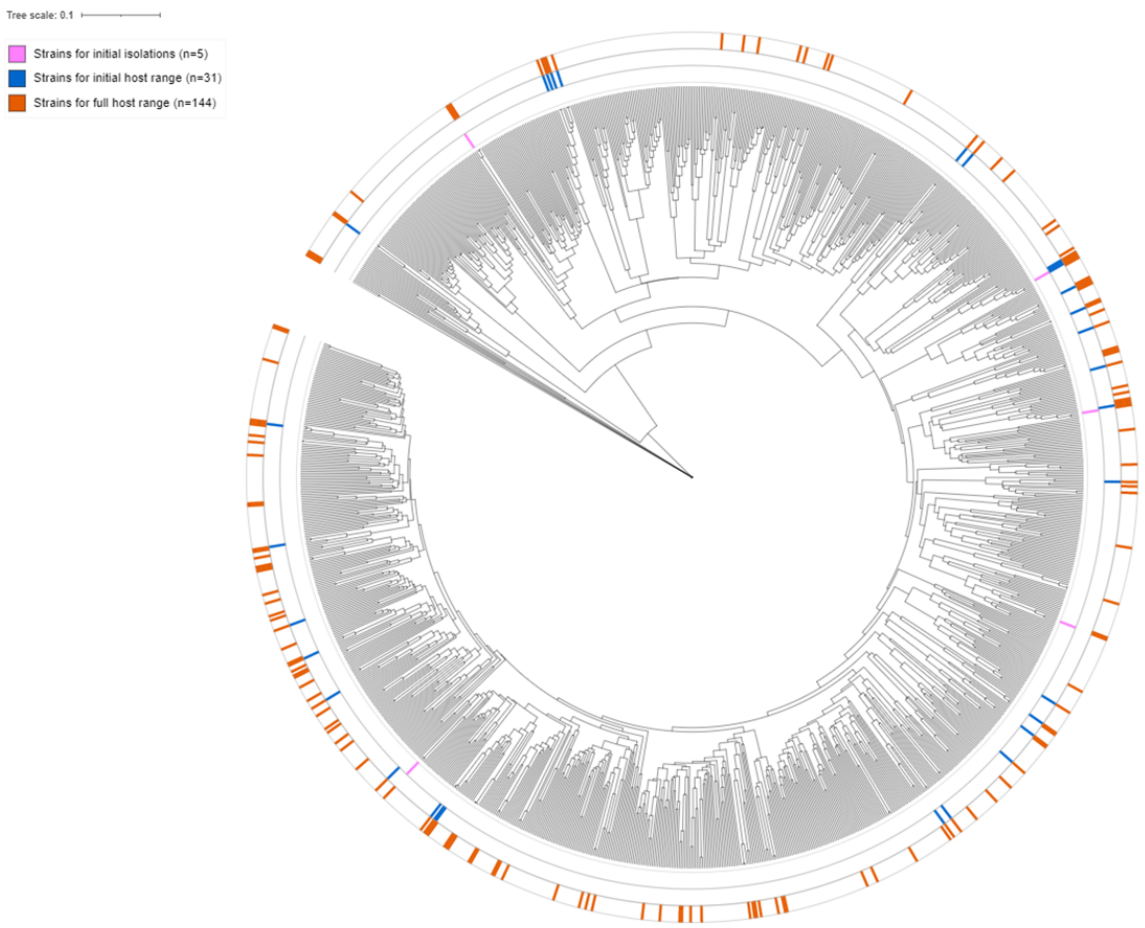

Figure S2. Tree visualization of genomic distances between RefSeq *P. aeruginosa* cluster representatives and the 143 clinical isolates in our panel. Source data are provided as a Source Data file.

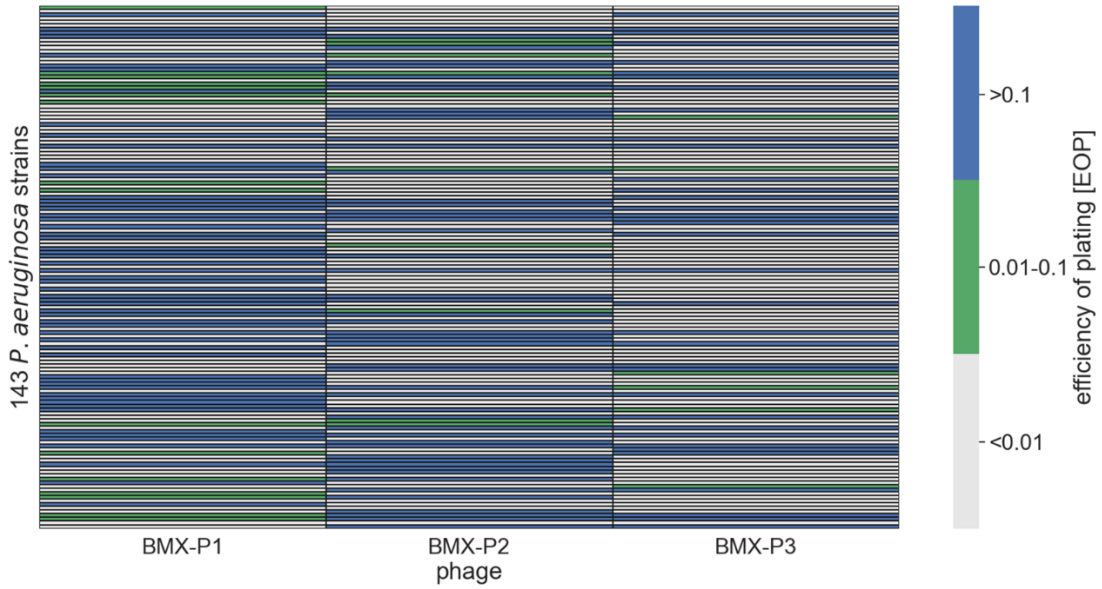

Figure S3. Full host range of BX004-A phages on 143 *P. aeruginosa* strains. Overall, 106 strains are susceptible to the BX004-A cocktail (74%), 74 strains are susceptible to BMX-P1 (52%), 62 strains are susceptible to BMX-P2 (43%), and 42 strains are susceptible to BMX-P3 (29%). 51 strains are susceptible to one phage, 38 strains are susceptible to two phages, and 17 strains are susceptible to all three phages. The high-level geographic breakdown of susceptibility to BX004-A is as follows: USA: 86/114 (75%), Europe: 17/26 (65%), Other: 3/3 (100%). Source data are provided as a Source Data file.

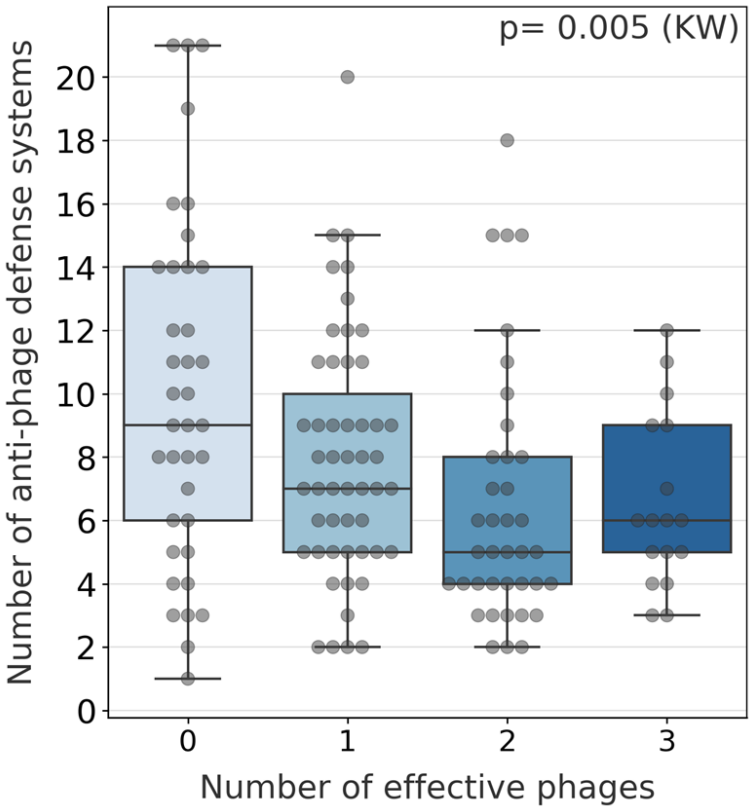

Figure S4. Number of phages that effectively lyse a strain vs. number of aPDS in the strain's genome. Kruskal–Wallis one-way analysis of variance yielded a p-value of 0.0054. Source data are provided as a Source Data file.

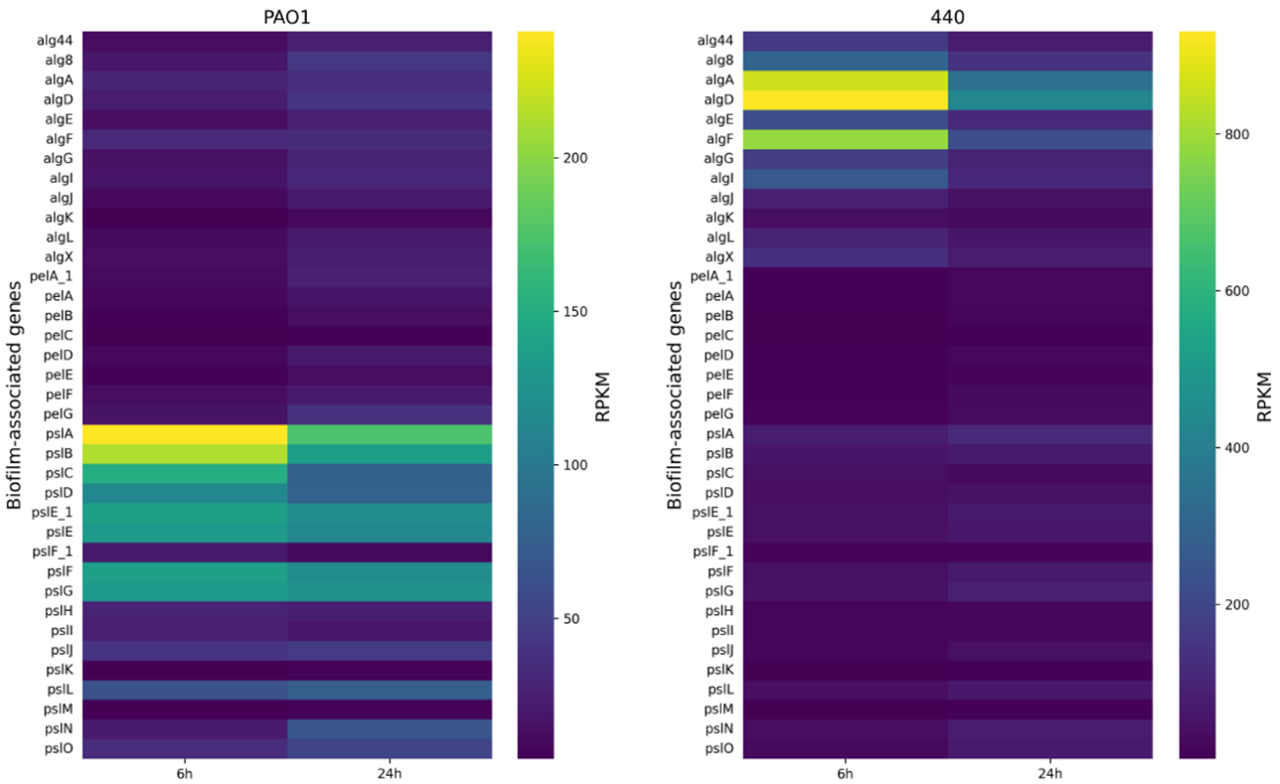

Figure S5. Relative transcript abundance (Reads Per Kilobase per Million mapped reads - RPKM) during biofilm formation, as determined by RNA-sequencing. The two strains (PAO1, 440) were grown as described in the methods ("Phage activity in biofilm"), and RNA was collected at two timepoints during growth: 6h and 24h following inoculation. RNA-seq was carried out as described in the methods ("Transcriptomics analysis"). The genes presented in this figure are all genes from the known *P. aeruginosa* biofilm pathways (Alginate, PEL, and PSL). Source data are provided as a Source Data file.

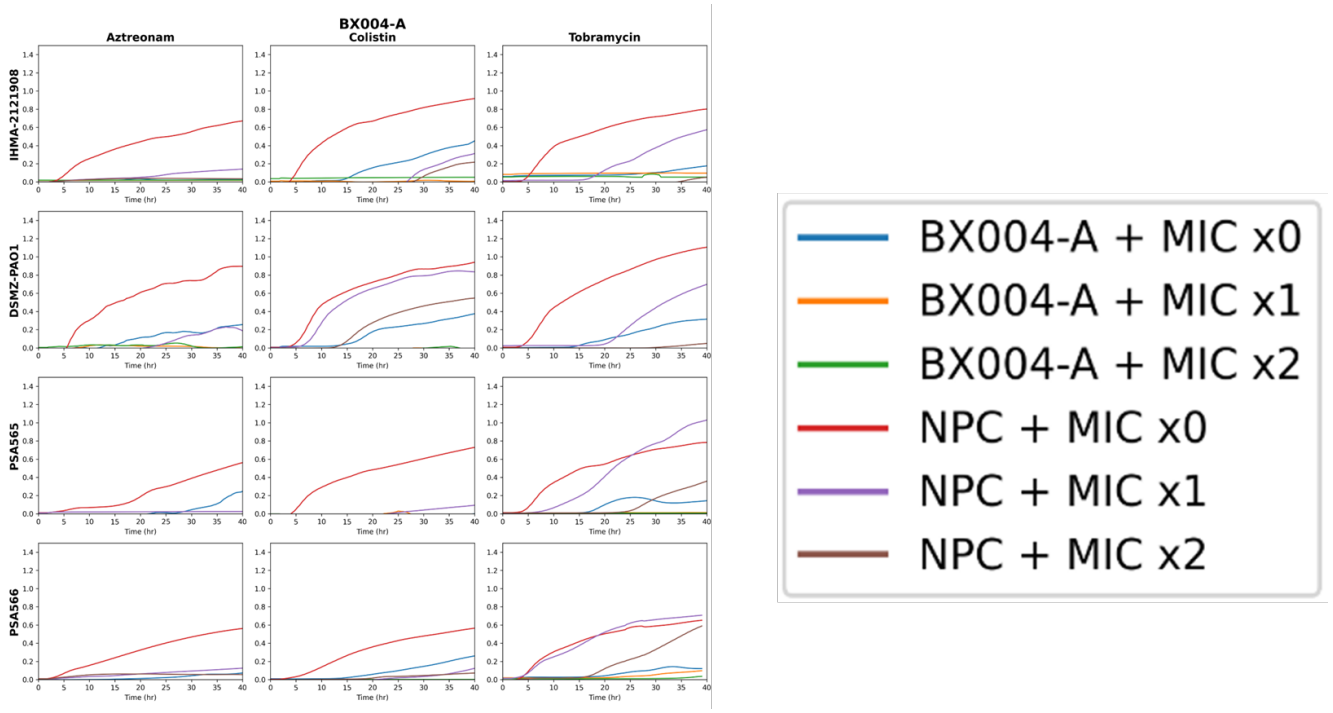

Figure S6. Growth curves depicting the growth dynamics of four distinct *P. aeruginosa* strains (y-axis) in the presence of three common antipseudomonal antibiotics used in CF (x-axis). "No phage" controls are marked as "NPC". "No antibiotic" control is marked as "MICx0". Source data are provided as a Source Data file.

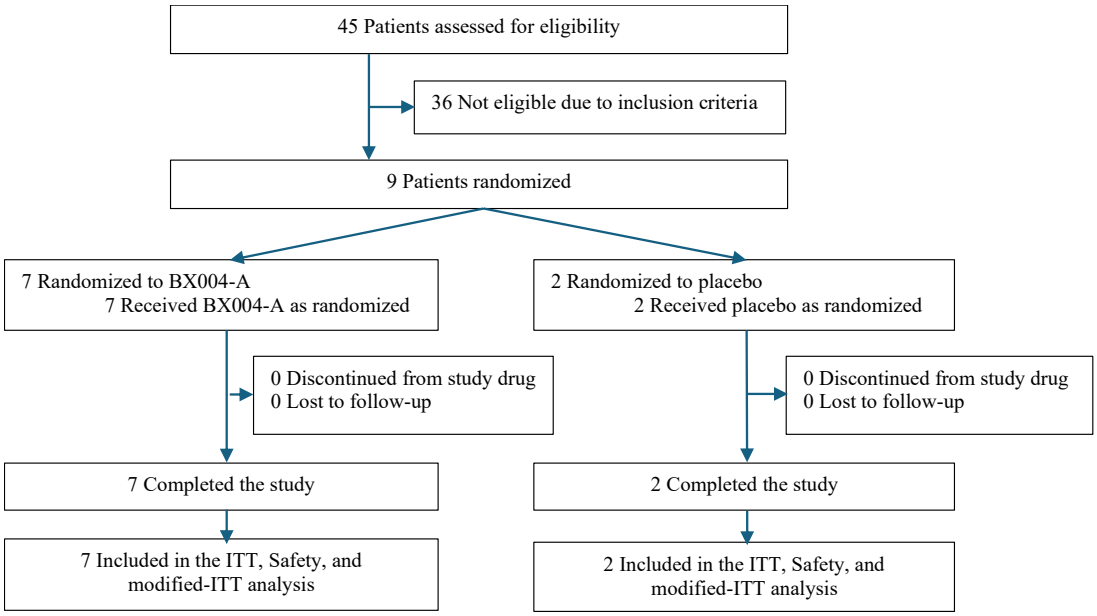

Figure S7. CONSORT diagram: 45 patients were assessed for eligibility, of which 9 were randomized. Seven patients received BX004-A and two received placebo vehicle. *Screen failure reasons: at least one phage-resistant *P. aeruginosa* morphotype found in sputum culture (n=19), insufficient *P. aeruginosa* levels (n=8), IV or oral antibiotics or corticosteroids in past 4 weeks (n=5), other (n=4).

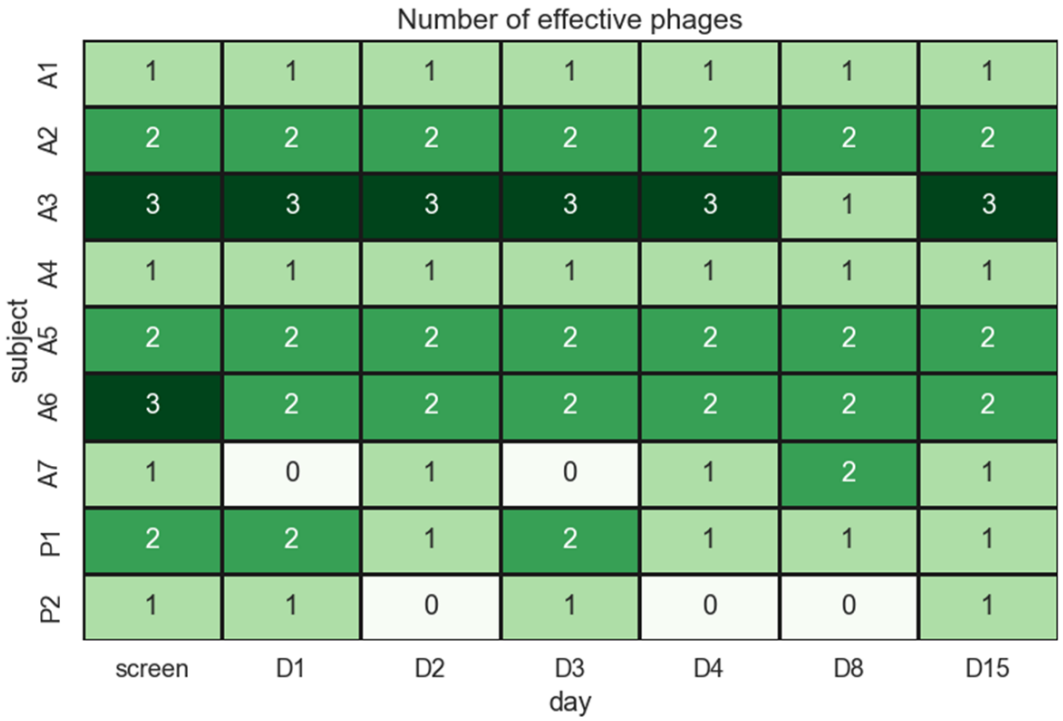

Figure S8. Number of effective BX004-A phages (EOP>0.1), as measured in vitro on *P. aeruginosa* strains isolated from patients' sputum longitudinally during the trial. Patients P1 and P2 received placebo. Patients A1-A7 received BX004-A. Source data are provided as a Source Data file.

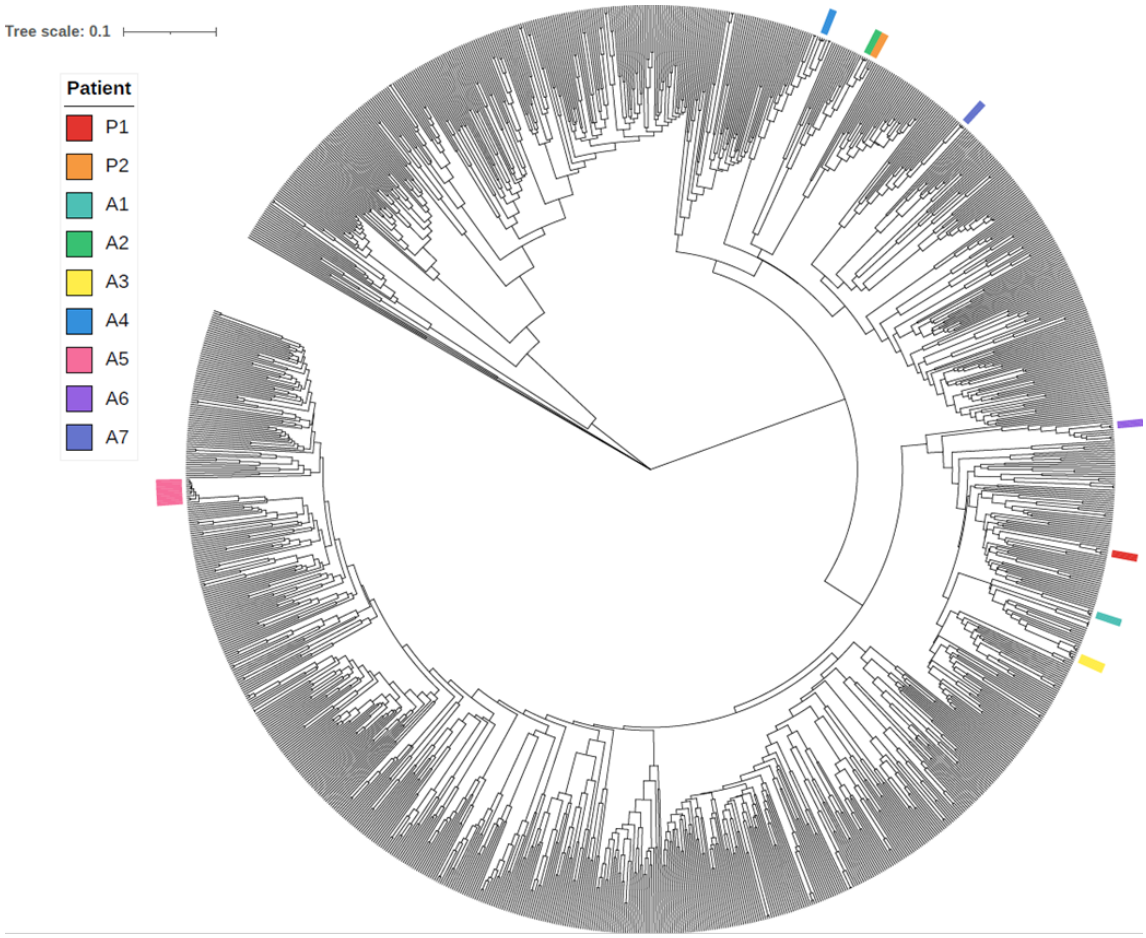

Figure S9. Genomic superimposition of strains collected from all trial subjects at screening, d1 and d15 on the *P. aeruginosa* species map depicted in Fig. 1A. Patients P1 and P2 received placebo. Patients A1-A7 received BX004-A.

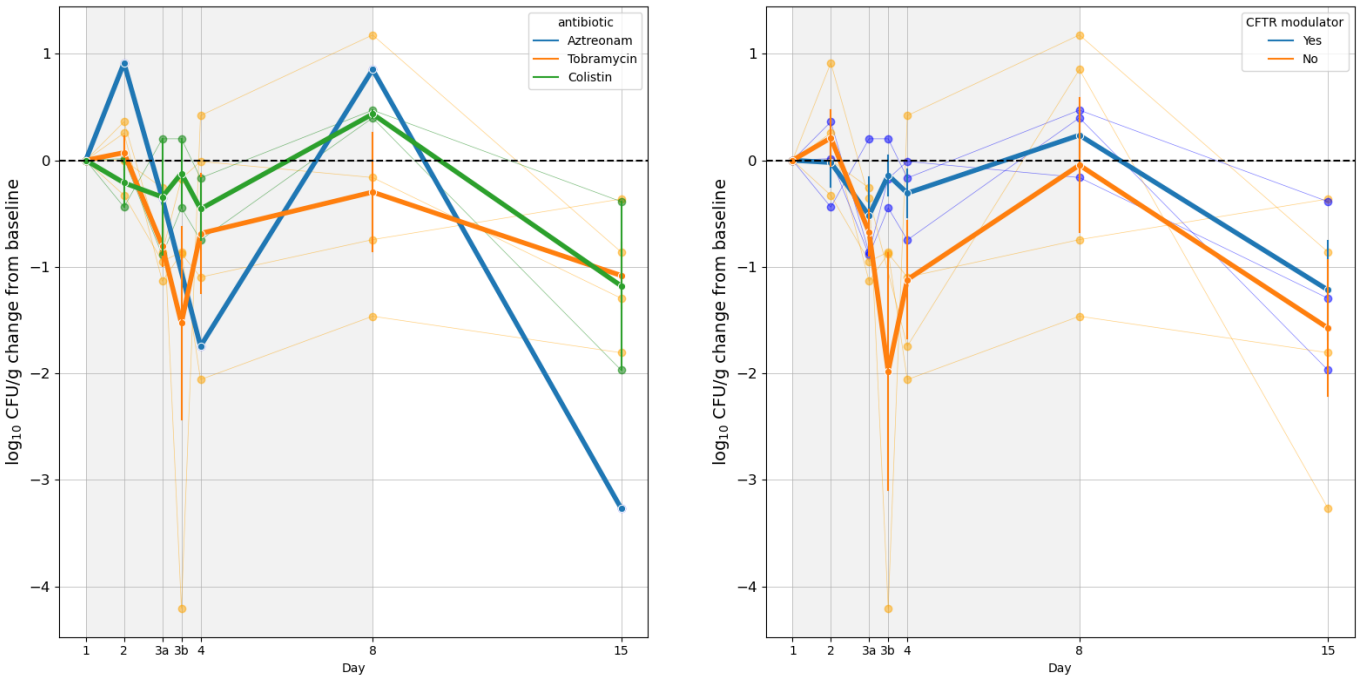

Figure S10. Post-hoc subgroup analysis of $\log_{10}$ CFU change from baseline within the BX004-A group alone (data obtained from patients in the placebo arm is not shown here). On the left, patients are grouped according to their standard of care inhaled antibiotic which they continued taking during the treatment. On the right, patients are grouped according to whether they were taking a CFTR modulator during the trial. Semi-transparent curves represent individual patients, whereas bold lines represent mean ± SE of the group. Source data (including detailed sample sizes) are provided as a Source Data file.

Table S1. **Novel lytic phages isolated**

GenBank accession	internal phage name	class	genus	species	genome size	# genes	GC%
OQ319930	VAR-THS-002-Wou-PA1-2	Caudoviricetes	Phikmvvirus	Unclassified	42999	62	62.1
OQ319931	VAR-THS-002-Wou-PA1-5	Caudoviricetes	Jamesmcgillvirus	Jamesmcgillvirus PaMx41	43217	56	45.3
OQ319932	BMX-P2	Caudoviricetes	Pbunavirus	Unclassified	65780	102	55.6
OQ319933	BMX-P3	Caudoviricetes	Pakpunavirus	Unclassified	90869	194	49.4
OQ319934	BMX-P1	Caudoviricetes	Bruynoghevirus	Bruynoghevirus PAA2	44725	81	52.5
OQ319935	VAR-THS-002-Wou-PA1-4	Caudoviricetes	Pbunavirus	Pbunavirus E217	66162	105	55.6
OQ319936	CCUG-53668-1	Caudoviricetes	Pakpunavirus	Unclassified	91543	194	49.4
OQ319937	CCUG-53668-2	Caudoviricetes	Pakpunavirus	Unclassified	91130	196	49.4
OQ319938	CCUG-53574-1	Caudoviricetes	Unclassified	Unclassified	62357	111	56.1

Table S2. **Nebulization PFU losses with final formulation**

type\ condition	**pre nebulization PFU**			**post nebulization PFU**			**mean loss [log10]**		
phage	BMX-P1	BMX-P2	BMX-P3	BMX-P1	BMX-P2	BMX-P3	BMX-P1	BMX-P2	BMX-P3
rep1	1.96E+09	1.01E+10	6.45E+09	1.12E+09	1.79E+09	3.08E+08	-0.31	-0.73	-0.92
rep2	2.13E+09	9.22E+09	1.15E+10	8.47E+08	1.69E+09	2.33E+09			
rep3	1.41E+09	8.97E+09	1.12E+10	7.11E+08	1.75E+09	2.03E+09			

PFU: plaque-forming units

Table S3. **Effective dose calculation**

phage	P3	P2	P1	BX004
nominal concentration [PFU/ml]	3.00E+09	3.00E+09	3.00E+09	9.00E+09
concentration post nebulization [PFU/ml]	3.69E+08	5.59E+08	1.47E+09	2.40E+09
concentration localized to ≤5µm aerosols [PFU/ml]	2.14E+08	3.24E+08	8.52E+08	1.39E+09
effective lung dose [PFU/dose]	3.43E+08	5.18E+08	1.36E+09	2.22E+09

PFU: plaque-forming units

Table S4. **Safety experiments conducted with final BX004-A product**

Type	Model	Dosing	Parameters tested	Observations
ex vivo	3D model of reconstituted human lung tissue (MucilAir™) derived from primary human airway epithelial cells of a CF patient and developed to simulate human-relevant biological responses	24 hour incubation with one of three concentrations of BX004-A (5*E5 PFU/mL, 5*E6 PFU/mL, and 5*E7 PFU/mL)	transepithelial electrical resistance (TEER), LDH release data and histopathology	No cytotoxicity or other abnormalities in any of the concentrations
in vivo	Male Balb/C mice	Repeated intranasal administrations once a day, for three days. Phage concentration (1.8*E8 PFU per administration) was the maximal dose that could be administered without concentrating the phage solution at the maximal volume that can be administered intranasally vs. a control group dosed with the formulation alone and a naive control group	Survival, morbidity, body weight, food consumption, organ weight, histopathology, hematology	Results showed that repeated intranasal administration of phage BX004-A mixture was not associated with any treatment-related toxicity; No animal was found in morbid condition or died during the study and no abnormalities in clinical signs were observed

Table S5. **Subject Baseline Characteristics**

Characteristic	**BX004-A (n=7)**	**Placebo (n=2)**
Age, mean, years (SD)	29.6 (6.6)	39.0 (19.8)
Male, n (%)	5 (71.4)	1 (50)
Female, n (%)	2 (28.6)	1 (50)
Israel, n (%)	6/7 (85.7)	2/2 (100)
US, n (%)	1/7 (14.3)	0/2 (0)
Race / Ethnicity: Caucasian (not Hispanic or Latino)	7 (100)	2 (100)
BMI (kg/m²), mean (SD)	22.8 (3.0)	24.6 (0.3)
Range	18.7 - 26.7	24.4 - 24.8
CFTR modulators	3 (42.9)	0 (0)
Inhaled antibiotic (during study drug):		
Aztreonam	1 (14.3)	2 (100)
Tobramycin	4 (57.1)	0
Colistin	3 (42.9)	0
% Predicted FEV1: < 40 %	0	0
40–49 %	2 (28.6)	0
50–59 %	2 (28.6)	0
60-69 %	1 (14.3)	0
>= 70 %	2 (28.6)	2 (100)
P. aeruginosa $\log_{10}$ CFU/g at D1; mean (SD)*	7.4 (1.5)	7.9 (0.1)
Range	4.2-8.5	7.8-8.0

*All subjects met Screening inclusion criteria of minimum *P. aeruginosa* in sputum at least 10^5 CFU/g

One subject switched from aztreonam (Days 1 through 5) to colistin (Days 6 through 8) due to supply and resumed aztreonam Days 9 through 15. Another subject switched from tobramycin to colistin on Day 9. All other subjects were on the same inhaled antibiotic from Days 1 through 15 (last sputum collection date).

BMI: Body mass index; CFTR: cystic fibrosis transmembrane conductance regulator; FEV1: forced expiratory volume in 1 second; CFU: colony-forming units; SD: standard deviation

Table S6. **Clinical safety results***

Parameter	**BX004-A (N=7)**	**Placebo (N=2)**
Completed study drug n/N (%)	7/7 (100)	2/2 (100)
Actual # of doses per subject, mean (SD)	11 (0)	11 (0)
Treatment Compliance, 100%, n/N (%)§	7/7 (100)	2/2 (100)
Number of adverse events (AEs)	9	1
Number of treatment-emergent adverse events (TEAEs)	9	1
Subjects with at least 1 TEAE, n (%)	4 (57.1)	1 (50)
Subjects with at least 1 TEAE related to study drug	0	0
Subjects with at least 1 TEAE leading to discontinuation of study drug, n (%)	0	0
Subjects with at least 1 TEAE leading to discontinuation of study, n (%)	0	0
Subjects with at least 1 serious adverse event (SAE), n (%)	1 (14.3)	0
Subjects with at least 1 TEAE leading to death, n (%)	0	0
Subjects with at least 1 TEAE by maximum severity		
Mild	0	1 (50)
Moderate	3 (42.9)	0
Severe	1 (14.3)	0
Common TEAEs (> 15% overall; preferred term) Infective pulmonary exacerbation of CF*	3 (42.9%)	0
AEs of special interest (AESI) Subjects with at least 1 Acute Pulmonary Exacerbation (by modified Fuchs criteria) Subjects with at least 1 Pulmonary Worsening After Study Drug Administration	0 0	0 0

§ Compliance is calculated as [100 x (# of full doses of study drug administered) / # of planned doses]

*Infective pulmonary exacerbation of CF occurred in long-term follow-up period and did not meet modified Fuchs Criteria: 1 SAE (52 days after last dose), 1 non-serious AE (133 days after last dose), and 1 non-serious AE (137 days after last dose); all recovered / resolved and were not related to study drug

Table S7. **Isolate MLST**

patient	timepoint	morphology	MLST
A1	screening	A	ST217
A1	d1	A	ST217
A1	d15	A	ST217
A2	screening	A	Novel (all alleles match ST308, besides mutL which is type 70)
A2	d1	A	Novel (all alleles match ST308, besides mutL which is type 70)
A2	d15	A	Novel (all alleles match ST308, besides mutL which is type 70)
A3	screening	A	ST116
A3	d1	A	ST116
A3	d1	B	ST116
A3	d15	A	ST116
A4	screening	A	ST253
A4	d1	A	ST253
A4	d15	A	ST253
A5	screening	A	ST2432
A5	screening	B	ST2432
A5	screening	C	ST2432
A5	d1	A	ST2432
A5	d1	B	ST2432
A5	d1	C	ST2432
A5	d1	D	ST2432
A5	d15	A	ST2432
A5	d15	B	ST2432
A5	d15	C	ST2432
A5	d15	D	ST2432
A6	screening	A	ST274
A6	d1	A	ST274
A6	d15	A	ST274
A7	screening	A	ST316
A7	d1	A	ST316
A7	d15	A	ST316
P1	screening	A	ST487
P1	d1	A	ST487
P1	d15	A	ST487
P2	screening	A	ST308
P2	d1	A	ST308
P2	d15	A	ST308

Table S8. **CFU change form baseline per treatment arm**

	Post-hoc analysis presented in figure 6B		Pre-specified analysis with adjusted baseline	
visit day	BX004-A - Mean (SE)	Placebo - Mean (SE)	BX004-A - Mean (SE)	Placebo - Mean (SE)
1	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
2	0.11 (0.16)	3.07 (NA*)	0.11 (0.16)	0.0 (0.0)
3a	-0.61 (0.17)	1.58 (1.37)	-0.61 (0.17)	0.05 (0.29)
3b	-1.06 (0.6)	-0.55 (0.0)	-1.06 (0.6)	-0.55 (0.0)
4	-0.77 (0.32)	1.15 (0.47)	-0.77 (0.32)	-0.38 (0.61)
8	0.08 (0.33)	0.65 (0.62)	0.07 (0.33)	-0.88 (0.47)
15	-1.42 (0.36)	1.26 (1.02)	-1.42 (0.36)	-0.27 (0.06)

The analysis presented in figure 6B is a post-hoc analysis of the data, as described below:
One of the two placebo patients had taken their standard of care inhaled antibiotic within 60 minutes of sputum collection on Day 1, which the statistical analysis plan pre-specified would lead to the Day 2 post-dose sputum sample being used as baseline CFU for any case where this applied, due to a potential interference with an accurate assessment of sputum CFU level (due to presence of antibiotic possibly reducing CFU ex vivo in the sample). This subject had a relatively low CFU measurement at Day 1 (6x10^4 CFU/g). When 16S data became available at a later stage, we observed an overall strong and significant correlation between *P. aeruginosa* relative abundance (measured based on 16S analysis) and CFU levels. We observed that in the aforementioned baseline sample that yielded a low CFU count, *P. aeruginosa* 16S relative abundance had a matching low relative value. Based on this orthogonal validation, we concluded that the low baseline CFU originally measured for this patient was a scientifically correct representation of P. aeruginosa density in the collected sputum sample at that timepoint, thus we decided to use it as the baseline value in all post-hoc analyses.

*There is only 1 datapoint at this time point, thus SE calculation is not applicable