The Diversity of a Polyclonal FluCell-SELEX Library Outperforms Individual Aptamers as Emerging Diagnostic Tools for the Identification of Carbapenem Resistant *Pseudomonas aeruginosa*

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Supplementary figures

Figure S1. Publications in NCBI Pubmed with the keyword „aptamer”

Figure S2. Confocal laser scanning microscopy of different medically relevant human pathogenic and commensal bacteria incubated with the polyclonal aptamer library R16.
Figure S3. Quality control of aptamer preparation for next generation sequencing by Illumina®. Enriched aptamers (lane 1) were extended by the introduction of a universal primer binding site (lane 2). In a next step index sequences were introduced to distinguish sequences present after one round of selection from sequences after the final selection round (lane 3).
Figure S4. Consensus sequences generated for the clustered aptamers. The possibility of consensus building within the given parameters demonstrates the plausibility of the determined aptamer clusters.
Figure S5. Stable quality of target recognition by the polyclonal R16 library on \textit{P. aeruginosa} cells upon repeated enzymatic amplification reactions resulting in four generations of descendants.

**Experimental Procedures**

**Materials**

The used ssDNA aptamer library with 40 randomized base pairs was purchased from TriLink Biotechnologies (San Diego, California, USA). 5'-Cy5-labelled forward primer ([Cy5]-TAGGGAAGAGAAGGACATATGAT) and biotinylated reverse primer (Biotin-TCAAGTGGTCATGTACTAGTCAA) were synthesized by Eurofins genomics (Ebersberg, Germany). Taq-DNA polymerase and dNTPs used for the PCR amplification were purchased from Qiagen (Venlo, Netherlands). For the alkaline strand separation of double stranded PCR products to generate single stranded aptamers, Dynabeads M-270 Streptavidin were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). NaOH, NaH$_2$PO$_4$, Tris, boric acid, ethylenediaminetetraacetic acid (EDTA), Rotiphorese gel 40, ammonium persulfate (APS) and N,N,N',N'-Tetramethylethylendiamin (TEMED) were purchased from Carl Roth (Karlsruhe, Germany) and appropriate stocks were prepared and stored at room temperature. Phosphate buffered saline (PBS) was purchased by life technologies (Carlsbad, California, USA). Statistical analysis was performed by two tailed unpaired student t-test where applicable. P values < 0.05 were considered significant. * denotes P < 0.05, ** < 0.01, *** P < 0.001 and **** P < 0.0001.

**Cultivation of bacteria**

In general \textit{Pseudomonas aeruginosa} and \textit{Escherichia coli} were grown 250 ml Erlenmeyer flasks containing 50 ml LB-medium (Carl Roth, Karlsruhe, Germany) at 37°C and 180 rpm. For the SELEX procedure 50 ml LB-medium were inoculated to an optical density OD$_{600nm}$ of 0.05. After reaching an OD$_{600nm}$ of 1 (= 10$^9$ cfu/ml$^{-1}$), cells were used for the incubation with aptamers. Alternatively cell growth was recorded through the increasing backscatter of the culture using a cell growth quantifier (Aquila Biolabs GmbH, Baesweiler, Germany) and growth rates were determined by the CGQuant software (Aquila Biolabs GmbH, Baesweiler, Germany). The mammalian commensals \textit{Akkermansia muciniphila} YL44 and \textit{Blautia producta} were cultivated without agitation in FAST medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37°C. The cultivation was thereby performed under anaerobic conditions (90% N$_2$/ 10% H$_2$) in a Whitley A35 anaerobic workstation (Meintrup DWS Laborgeräte GmbH, Herzlake, Germany). Like \textit{P. aeruginosa} and \textit{E. coli}, \textit{Streptococcus agalactiae} was cultivated in BHI medium (Carl Roth, Karlsruhe, Germany) at 37°C and 180 rpm.

**Electrophoretic methods**
Electrophoretic separation of DNA samples were analyzed in 2% agarose gels. TBE (44 mM Tris, 44 mM boric acid, 1 mM EDTA, pH 8.3) buffer was thereby used as solvent for the agarose and as running buffer. Separation was performed at 125V for 45 min in a horizontal PerfectBlue® gel system (Pqelab, Erlangen, Germany). Gels were subsequently stained in a 0.0001% Ethidium bromide (Carl Roth, Karlsruhe, Germany) solution and visualized in an E-gel imager with a UV light base (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Proteins were electrophoretically separated according to well established protocols according to Lämml[2], in short, a 12% acrylamide gel was prepared after appropriate dilution of a 40% acrylamide solution (Rotiphoreese gel 40, Carl Roth, Karlsruhe, Germany, acrylamide: Bis-acrylamide = 37.5:1) in 1.5M Tris (pH 8.8) and addition of SDS to 0.1%, in a radical polymerization induced by the addition of 0.1% APS and 0.1% TEMED. The separating gel was overlaid with a 5% stacking gel, which was prepared accordingly using 1M Tris (pH 6.8). After electrophoretic separation in a vertical PerfectBlue® gel system (Pqelab, Erlangen, Germany) at 30 mA, gels were stained in a colloidal coomassie G-250 solution (0.4% Coomassie brilliant blue G-250, 5% aluminum sulfate, 10% ethanol, 2% orthophosphoric acid) as earlier described [3].

SELEX procedure

At the beginning of each selection round the aptamers were heated to 95°C for 5 min, snap cooled on ice and equilibrated to room temperature to ensure correct folding of the aptamer. In the initial round of selection 1 nmol of the aptamer library was incubated with approximately 2x10^8 P. aeruginosa PA01 cells in PBS. After 60 min of incubation at room temperature on a rotator (50 rpm) cells were centrifuged at 8000 x g for 2 min and the supernatant was discarded. The cells were then washed in 500 µl PBS and finally resuspended in 100 µl PBS. For elution of the bound aptamers the resuspended cells were heated to 95°C for 5 min and immediately centrifuged at 8000 x g for 2 min. The supernatant was then separated into 20 µl aliquots, which served as the template in the following PCR amplification. The PCR was then performed in 1x PCR buffer (1.5 mM MgCl2) containing 200 µM of each dNTP, 0.5 µM of Cy5-labelled forward and biotinylated reverse primer. The amplification took place in a SensoQuest Labcycler (SensoQuest GmbH, Göttingen, Germany) in a three-step reaction including an initial denaturation for 3 min at 94°C, 25 cycles of 94°C for 30 sec, 49.1°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 2 min. The double stranded PCR products were then pooled and incubated with 250 µg Biomag Streptavidin beads (Qiagen, Venlo, Netherlands), which were previously washed three times with 1 ml PBS, on a rotator (50 rpm) at room temperature for at least 1 h. The alkaline strand separation was then achieved by resuspending the beads in 50 µl of a 100 mM NaOH solution. After 5 min incubation the supernatant was transferred to 125 µl PBS and the pH was neutralized by the addition of 43 µl 100 mM NaH2PO4. The concentration of this aptamer pool was measured using a Nanodrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and adjusted with PBS to the desired concentration.

To increase the selective pressure throughout the SELEX process several measures were taken. Already in the second round of selection the aptamer concentration was lowered to 10 pmol. From round 3 to 8 the aptamer concentration was kept at 3 pmol and from round 9 to 16 at 1 pmol. Furthermore, the incubation time was decreased from 60 min during the first two rounds, to 45 min in the third round, to 30 min from the fourth round. Additionally, the number of washing steps was increased to three steps and a counterselection against E. coli DH5a was introduced from round 9. For the counterselection the prepared aptamer library was preincubated with 10^8 E. coli DH5a cells for 1 h on a rotator at 50 rpm. Cells were then removed by centrifugation at 8000 x g for 2 min and the supernatant was transferred to the P. aeruginosa cells for positive selection.

Combined analysis of aptamer binding

The evolution of specificity of aptamers in the described cell SELEX was followed by fluorescence based assays (FluCell-SELEX). Therefore 10 pmol of Cy5 labelled aptamers from selection rounds 2, 5, 8, 13, 15 and 16 were incubated with 2 x 10^8 P. aeruginosa PA01 cells for 30 min at room temperature on a rotator (50 rpm). Cells were then removed by centrifugation (2 min, 8000 x g) and washed three times in 500 µl PBS. The further analysis was performed directly by confocal laser scanning microscopy or by fluorescence measurements.

For confocal laser scanning microscopy a Zeiss Confocal Microscope (Carl Zeiss Ag, Oberkochen, Germany) was used. The Cy5-labelled aptamers were excited at a wavelength of 635 nm and the detected fluorescence was merged with the transmitted light image using Zen software (Zen 2012 Sp1, black edition, version 8.1.0.484), which was used to locate the bacterial cells.

For fluorescence measurements the elution of bound aptamers was performed by resuspending the cells in 100 µl PBS and heating to 95°C for 5 min. After an immediate centrifugation for 2 min at 8000 x g the supernatant containing the bound aptamers was saved and fluorescence was measured at an excitation wavelength of 635 nm and an emission wavelength of 670 nm using a Tecan infinite M200 plate reader (Tecan group AG, Männedorf, Switzerland). In this approach also the binding constants of R16 and C1R1 and the limit of detection (LOD) were determined by varying the aptamer concentrations or P. aeruginosa cell number respectively. The binding constants (k0) were calculated from a plot of the measured fluorescence (F) against the aptamer concentrations (c) fitted by a Hill equation $F = F_{max} * c^n / k_0^n + c^n$ with n as the Hill coefficient as a measure of binding cooperativity. The LOD was estimated from a plot of the fluorescence intensities against decreasing cell numbers. The limit of detection was represented by three standard deviations from the mean blank values which contained no P. aeruginosa cells. The same protocol was applied to also check the binding to the counter selection strain E. coli DH5a and to investigate the potential of a fluorescently labelled aptamer library to specifically retrace P. aeruginosa.
**Supporting Information**

*aeruginosa* cells in different mixtures containing 30, 60, 90%, pure or no *P. aeruginosa* cells, as well as to detect clinical *P. aeruginosa* isolates including a class 4 multi resistant strain. Clininal strains of *Pseudomonas aeruginosa* were identified by automated mass spectrometry (MALDI-ToF) using standard procedures at the Institute of Medical Microbiology at the University Hospital Ulm. Minimal inhibitory concentrations were determined by microdilution-based automated VITEK (Biomerieux) and confirmed by e-testing (Biomerieux). Expression of the VIM carbapenemase was confirmed by automated light cycler PCR (Becton Dickinson). Bacterial strains were collected from patient samples sent to the Microbiology Department for diagnostic purposes. Strains were collected anonymously and it is not possible to track the source of the bacteria. The accreditation number of the Microbiology Department is DIN EN ISO 15189:2014 (DAkkS).

The precision of detection was calculated as the absolute value of the difference subtracted from 100% relative to the *P. aeruginosa* PAO1 binding of R16 and C1R1. (Precision [%] = 100% - |F(PAO1, %) - F(Clin, %)|)

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**Next generation sequencing and identification of single aptamer sequences**

The final round 16 and the first round of the SELEX process were prepared for Illumina® sequencing by GATC Biotech (GATC Biotech AG, Konstanz, Germany). As this method involves bridge amplification, the aptamers were prolonged to allow a sufficient bridge formation. This was done by two PCR steps. The first PCR was performed using the forward primer 5'-ACGATGATACCTCGGACTGTAGGGAAGAGAGCATATGAT-3' and the reverse primer 5'-TCTCGTAGTTCAAGC GACTCAATGTGCTATGTACTAGTCAACA-3' to introduce universal primer binding sides (underlined). The PCR was performed using a Phusion® DNA polymerase (NEB Inc., Ipswich, Massachusetts, USA) according to the manufacturers protocol in a three-step thermal reaction: initial denaturation for 3 min at 94°C, 9 cycles of 94°C for 30 sec, 49.1°C for 30 sec and 72°C for 30 sec, and a final extension for 2 min at 72°C. The extended double stranded aptamers were then purified with a QiAquick PCR purification kit (Qiagen, Venlo, Netherlands) and served as a template for the second extension, during which index sequences were introduced to allow a parallel sequencing of aptamers from round 1 and 16. For the extension of aptamers from round 1 the primers 5'-TCAGTCGTATATCAGACC ATGATACCTCGAAGCTG-3' and 5'-GCTATGTACTCGTATTCTCGTAGTCAAGCGAC-3' were used, while aptamers from round 16 were extended with the primers 5'-TCACCTCGATATCGATACGATACCTCGGACTG-3' and 5'-GCTATGTACTACATCGTCT CGTAGTTCAGCGAC-3'. The indices introducing sequences, which were chosen according to published recommendations [8], are written in bold. The PCR was performed equally to the first extension with the exception that an annealing temperature of 56.6°C was used. The quality of the NGS data was checked with FastQC®. Further preprocessing including the sorting of the sequences based on the introduced indices as well as the clipping of the primer binding sites and analysis of the nucleotide distribution was done by using the FASTX toolkit [8]. Finally the sequences were analyzed with the FASTAptamer toolbox [7], which included the analysis of the enrichment and clustering of the sequences. Consensus sequences were built using CLC workbench with a gap open cost of 1, gap extension cost of 1 and a free end gap cost. The possibility of the consensus sequence building gave a direct readout for the plausibility of the clustered sequence families. However the secondary structure of the determined sequences was then predicted by using the Mfold server for a folding temperature of 25°C and a Na+ concentration of 137 mM [9] and revealed structural differences in the consensus sequence and the actual leading sequences pointing out the importance of individual sequences.

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**Motility assay for P. aeruginosa PAO1**

The swimming motility of *P. aeruginosa* was investigated accordingly to Wilhelm et al [5]. In short, a single colony of *P. aeruginosa* PAO1 was staked on top of a PPGAS swimming agar plate (20 mM NH₄Cl, 20 mM KCl, 120 mM Tris-HCl, 1.6 mM MgSO₄, 0.5% [wt/vol] glucose, 1% [wt/vol] peptone, 0.3% [wt/vol] agar; adjusted to pH 7.2). In close proximity (2-3 mm) to the cells the agar was holed using a 1 ml pipette. This hole was filled with 5 μl aptamer solution containing 1 to 10 pmol aptamer. Unspecific single stranded herring sperm DNA (Sigma-Aldrich, St. Louis, USA) at an analog concentration and PBS only served as a control.

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**Testing of biofilm formation in the presence of aptamers**

To investigate the motility of the initial biofilm formation, an assay based on the protocol of O’Toole was performed in a 96 well plate [10]. In short, an overnight culture *P. aeruginosa* PAO1 (OD600nm ~ 3) was diluted 1:100 into fresh TSB medium (Carl Roth, Karlsruhe, Germany). 10 pmol of aptamers were added to the cells and incubated without agitation at 30°C for 30 min, to analyze initial attachment of the cells. To exclude unspecific effects of single stranded DNA per se, 280 ng single stranded herring sperm served as control. After incubation wells were washed with water and 125 μl of 0.1% crystal violet was added. After 15 min of incubation at room temperature and a further washing step with water, the plate was air-dried overnight. The next day, the crystal violet stain was solubilized in 125 μl 30% acetic acid and quantified by measuring the absorbance at 550 nm.

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**Examination of labeling fidelity towards a growing culture**
Pull down assay for the analysis of aptamer library targets

For the analysis of the targets of the aptamer library, membrane extracts from *P. aeruginosa* were produced according to a simplified protocol modified from Hancock et al. In short, 0.8 g cells from an overnight culture were resuspended in 10 ml 30 mM Tris + 20% sucrose and incubated with 14 U of benzonase and 14 mg of lysozyme for 10 min at room temperature. After subsequent sonication (6x 1 min, 60%), the sample was centrifuged for 10 min at 4000 xg. The supernatant was diluted with 14 ml 30 mM Tris + 20% sucrose and centrifuged for 2 h at 25000 rpm and 4°C. The resulting pellet was then resuspended in 2.5 ml 30 mM Tris, 1% sodium cholate (w/v) and used as membrane fraction. 1.5 mg carboxylic acid modified M270 magnetic beads (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were washed twice using 50 µl 25 mM 2-(N-morpholino)ethanesulfonic acid (pH 5.0, MES). The beads were then resuspended in 60 µl 25 mM MES containing 2.5 mg/ml 5’-amino modified aptamers from R16 and incubated for 30 min at 25°C. After that 15 µl of a freshly prepared 100 mg/ml 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide in cold 100 mM MES (pH 5.0) were added and the mixture was filled up to 100 µl with 25 mM MES. The coupling was then performed for 16 h at 4°C under slow tilt rotation. To block remaining activated groups, the beads were incubated for 30 min in 100 µl 100 mM Tris (pH 8.0) at 25°C. The modified beads were then washed twice with 200 µl of the modified beads were heated to 95°C and cooled down to room temperature for activation and then incubated with 200 µl of the membrane fraction for 16h under slow tilt rotation. Beads were washed twice, using 200 µl DPBS and analyzed in a 12% SDS gel. Bands of interest were excised and analyzed employing an U3000 RSLCnano (Thermo Fisher Scientific, Idstein, Germany) coupled to an LTQ Orbitrap Velos Pro (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer as described previously with the exception of using the UniProt pseudomonas database for protein identification (www.uniprot.org).

Enzymatic production of polyclonal aptamer libraries by successive reamplification

The possibility of reusing an evolved aptamer library was investigated by consecutive reamplification of aptamer libraries originating from the enriched aptamer pool from SELEX round 16. The reamplification and strand separation was performed accordingly to the previously described protocols. Thereby 10 ng of the reamplified PCR products served as a template for consecutive reamplification while the remaining PCR products were purified by strand separation to generate a new round 16 aptamer library. The binding of the libraries, which were produced this way, was examined as described above at equimolar concentrations and equal *P. aeruginosa* cell amounts.

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Author Contributions

Dennis Kubiczek acquired most of the experimental data, drafted the manuscript, composed the figures and did final editing.
Heinz Raber acquired experimental data of clinical isolates, composed figures and was involved in the final editing of the manuscript.

Nicholas Bodenberger acquired confocal laser scanning microscopic data and prepared figures.

Thomas Oswald and Melis Sahan were involved in different aspects of the experimental work including cultivation of *P. aeruginosa*, molecular genetics and biochemical analysis.

Daniel Mayer supervised experiments in the safety lab for the cultivation of clinical isolates.

Sebastian Wiese was responsible for mass spectrometry and analyzed the resulting data.

Steffen Stenger selected and characterized clinical strains, was involved in scientific discussions and critically edited the manuscript.

Tanja Weil consulted the design of the study, interpreted data and helped editing the manuscript.

Frank Rosenau initiated and coordinated the study, interpreted data and finalized the manuscript.