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Prevalence of bacteriocins and their co-association with virulence factors within *Pseudomonas aeruginosa* catheter isolates

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Annotation

Urinary tract infections caused by *Pseudomonas aeruginosa* present a serious complication in the context of urinary catheter use. *P. aeruginosa* are equipped with various virulence determinants that may result in increased resistance. That contributes to higher morbidity and mortality in vulnerable patients. Pyocins are proteinaceous agents produced by pseudomonads with an antimicrobial effect targeting closely related bacteria. In this study, we examined the inhibition interactions among a set of 135 *P. aeruginosa* clinical isolates originated from the catheter-associated urinary tract infections. Togerther with pyocinogeny, the co-occurrence of other virulence factors and their co-association with pyocins were examined. The strong antagonistic interactions and high prevalence of pyocin genes among the isolates were observed. The overall competitive interactions provide promising findings in the area of alternative antibiotic approaches.

Declaration [in Czech]

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V Brně dne 26.2.2021 Mgr. Kristýna Dufková

Přiznání spoluautorství

Svým podpisem potvrzuji plnohodnotné spoluautorství Mgr. Kristýny Dufkové na článku: Prevalence of bacteriocins and their co-association with virulence factors within *Pseudomonas aeruginosa* catheter isolates (IF= 3,113; 2019). Mgr. Kristýna Dufková se v rámci této publikace podílela na fenotypové detekci inhibičních látek, provedla PCR detekci pyocinových genů, podílela se na zpracování a vizualizaci výsledků a připomínkovala finální podobu manuskriptu.

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Prevalence of bacteriocins and their co-association with virulence factors within *Pseudomonas aeruginosa* catheter isolates

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ABSTRACT

Urinary tract infections represent common nosocomial infectious diseases. Bacteriocin production has been recently described as a putative virulence factor in these infections but studies focusing particularly on *Pseudomonas aeruginosa* are not available. Therefore, we assessed the prevalence of the bacteriocin genes, their co-occurrence and their co-association with previously detected virulence factors in a set of 135 *P. aeruginosa* strains from catheter-associated urinary tract infections (CAUTIs). The overall bacteriocinogeny reached 96.3 % with an average of 3.6 genes per strain. The most frequently detected determinants were the encoded pyocins S4 (76.3 %), R (69.6 %), and S2 (67.4 %). A statistically significant co-occurrence and a negative relationship were observed between several pyocin types. Particular pyocins exhibited associations with biofilm formation, production of pyochelin, pyocyanin, antibiotic-degrading enzymes, overall strain susceptibility and resistance, and motility of the strain. Co-occurrence of the pyocins S2 and S4 ($p << 0.0001$; $Z = 13.15$), both utilizing the ferripyoverdine receptor FpvAI, was found but no relation to pyoverdine production was detected. A negative association ($p = 0.0047$; $Z = -2.83$) was observed between pyochelin and pyocin S5 utilising the ferripyochelin receptor FptA. Pairwise assays resulted in 52.1 % inhibition which was equally distributed between soluble and particle types of antimicrobials. In conclusion, pyocin determinants appear to be important characteristics of CAUTI-related *P. aeruginosa* isolates and could contribute to their urovirulence.

1. Introduction

Pseudomonas aeruginosa, a Gram-negative bacterium, is an opportunistic human pathogen connected with significant morbidity and mortality of vulnerable patients. The vast metabolic plasticity of the strains enables bacterium to colonize various abiotic surfaces and artificial medical devices as well as the host's tissues (Murray et al., 2007). According to worldwide clinical data, *P. aeruginosa* is responsible for approximately 20 % of all nosocomial infections (Driscoll et al., 2007). Owing to an intrinsically resistant phenotype, an ability to form biofilms and the capacity to produce a range of tissue damaging extracellular products, *P. aeruginosa* is capable of causing infections in various tissues, *e.g.*, the respiratory tract, the bloodstream, or the urinary tract (Mittal et al., 2009; Stehling et al., 2008).

Urinary tract infections (UTIs) belong to the most frequent nosocomial infections; *P. aeruginosa* represents one of the common etiologic agents covering around one-tenth of these infections (Newman et al., 2017). The insertion of indwelling urethral catheters is connected to the vast majority of UTIs (Purvis et al., 2014; Weber et al., 2011). The pathogenesis of *P. aeruginosa* catheter-associated urinary tract infections (CAUTIs) is multifactorial and involves both cell-associated and secreted virulence determinants (Mittal et al., 2009). Some virulence factors (proteases, phospholipase C or siderophores production) appear frequently in uropathogenic *P. aeruginosa* strains (Hamood et al., 1996; Mittal et al., 2009; Woods et al., 1997) and co-occurrence of particular virulence factors in CAUTI-related *P. aeruginosa* was described previously (Olejnickova et al., 2014).Whilst several studies have described the role of bacteriocin production in the pathogenesis of UTIs in *E. coli* strains (Azpiroz et al., 2009; Smajs et al., 2010), no such data are available for *P. aeruginosa.* Moreover, co-association of bacteriocin

Abbreviations: CAUTI, catheter-associated urinary tract infection; CF, cystic fibrosis; ESBL, extended spectrum β-lactamase; MBL, metallo-β-lactamase; UTI, urinary tract infection.

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production with other virulence factors or with the type of infection has not been investigated in *P. aeruginosa*.

Bacteriocins represent various groups of proteinaceous antimicrobials, those produced by *Pseudomonas* spp. are referred as pyocins (Michel-Briand and Baysse, 2002). Colicin-like (S-type and M-type) pyocins are characterized by modular organization; protein harbours receptor-binding, translocation and a toxin domain (Cascales et al., 2007). The toxin domain can be equipped by a nuclease, pore-forming or cell wall-degrading activity (Barreteau et al., 2012; Michel-Briand and Baysse, 2002). L-type pyocins contain tandem lectin domains with specificity to D-rhamnose in the cell envelope (Ghequire and De Mot, 2014; McCaughey et al., 2014), but the exact killing mechanism is not clear. Tailocins are high-molecular-mass bacteriocins resembling phage tail-like particles (Michel-Briand and Baysse, 2002). R-type pyocins are rigid and contractile structures corresponding to the tail of Myophages, whereas F-type pyocins are flexible, non-contractile particles resembling Siphophages (Nakayama et al., 2000). Tailocins generate depolarization of the cytoplasmic membrane (Fernandez et al., 2017; Ge et al., 2015). Soluble pyocins are encoded by a single killing gene, usually followed by a self-immunity conferring gene, whereas tailocins' gene clusters harboured around 15 genes per particle (Ghequire and De Mot, 2014).

In the present study, we investigated the prevalence of pyocin genes in a collection of CAUTI-related *P. aeruginosa* strains, their co-occurrence among the strains and their co-association with previously detected virulence factors (see paper Olejnickova et al., 2014). Finally, phenotypic manifestation of the pyocin genes was conducted through directional inhibition assays, which determined the overall competitive interactions and outlined the possible involvement of pyocin production in intra-species interactions in CAUTIs.

2. Material and methods

2.1. Bacterial strains

Pseudomonas aeruginosa strains used throughout this study originated from patients suffering from a CAUTI. A total of 135 strains were collected from St. Anne's University Hospital, Brno, Czech Republic, Centre of Cardiovascular and Transplantation Surgery, Brno, Czech Republic, Masaryk Memorial Cancer Institute, Brno, Czech Republic and Brothers of Charity Hospital, Brno, Czech Republic. The strains were identified using commercial biochemical tests (NefermTEST and OXItest, Lachema, Brno, Czech Republic) and species affiliation was confirmed by MALDI-TOF MS analysis. A MALDI Biotyper with Flex Control 3.4 software (Bruker Daltonik, Billerica, MA) was used according to the manufacturer's instructions. The manufacturer-recommended cut-off scores were used for identification, with scores of $\geq$2.000 indicating identification to the species level, scores between 1.700 and 1.999 indicating identification to the genus level, and scores of $<$1.700 indicating no identification. For PCR detection, previously referred *P. aeruginosa* producers were used as positive controls: strain NIH-H for pyocin S1 (Sano et al., 1993), strain PAO1 for pyocin S2 (Sano et al., 1993), pyocin S4 (Elfarash et al., 2012), pyocin S5 (Ling et al., 2010) and a conserved part of tailocins (pyocin R and F, Nakayama et al., 2000), and strain C1344 for pyocin L (McCaughey et al., 2014). Positive controls for the remaining pyocin types were obtained during the study from the set of clinical isolates (strain 64AC for AP41 and S3, strain 57CE for pyocin M1, and strain 09EA for pyocin M4); the corresponding PCR products were subsequently sequenced and compared to the references.

2.2. Detection of pyocin genes

The ocurrence of pyocin genes was investigated by colony PCR using the primers listed in Tab. S1. Genomic DNA was prepared from 3 to 5 fresh colonies by their resuspendation in 500 μL of distilled water and heating at 94 °C for 15 min. The cell debris was removed by centrifugation (7500 rpm for 15 min). The presence of soluble pyocins (AP41, S1–S5, L, M1, M4) and tailocin gene clusters was assessed using Taq 2x Master Mix (New England Biolabs, USA). Primers targeting a conservative part of the tailocin clusters (ehomologues of PA0614 and PA0632 for detection of pyocin R and F) was used according to Bakkal et al., 2010. The following PCR protocol was applied: 95 °C (5 min); 95 °C (30 s), 55 °C (30 s), 72 °C (1 min), 30 cycles; 72 °C (7 min). Harbouring of particular pyocin gene combinations was grouped into the inhibition haplotypes according to previous studies (Bakkal et al., 2010; France and Remold, 2016).

2.3. Sanger sequencing

PCR amplicons were sequenced by the ABI PRISM BigDye terminator v3.1 Cycle sequencing kit and ABI PRISM 3130 sequencer using the same primers as for the PCR analysis. The obtained reads were manually trimmed to remove low quality data which resulted in their final length between 137–167 bp. The amplicons were compared with the reference sequences of pyocin S1 (GenBank acc. no. D12707.1), pyocin S3 (X77996.1), pyocin M1 (AXRE01000014.1:5266−6135) and pyocin M4 (AXPZ01000017.1:71740−72768) using the blastn algorithm (Zhang et al., 2016). Nucleotide identity of the PCR products and references exceeded 97.5 % (data not shown).

2.4. Pairwise inhibition assay

The overall inhibition interactions among the strains were tested using a double-layer plate assay (Smarda et al., 2007). In total 89 isolates were screened in an all-by-all set up, *i.e.*, each strain was used as an indicator and as a potential producer. A producer strain was inoculated into the Tryptone Soya Agar (Oxoid, Great Britain) supplemented with mitomycin C (Accord Healthcare Limited, UK; final concentration 20 μg/mL) and cultivated at 37 °C for 24 h. The cells were killed by chloroform vapours and the plate was overlayed with a top TSA agar layer (0.7 %, w/v) containing 10^7 cells of an indicator culture. The inhibition zones were assessed after 24 h cultivation at 37 °C and scored according to the dimension of the inhibition zone; a small (1–2 mm) zone suggesting tailocin production whereas a large diffuse zone could be observed in S-type, L-type, and M-type pyocins producers (France and Remold, 2016; Fyfe et al., 1984). Mitomycin C addition increased pyocin production and reproducibility of the inhibition assay (France and Remold, 2016; Smarda et Benada, 2005) therefore the assay was performed only once.

2.5. Statistical analysis

The data were tested for normality where such testing was necessary. As most of the data were non-parametric, a complex statistical analysis was performed using non-parametric tests. Correlations between the pairs of pyocins and pairs of pyocins with production of virulence factors, and clinical and patient-related data were analysed separately by the Statistica software for Windows 12 (StatSoft, Inc., 2013), using the Goodman-Kruskal correlation coefficient γ. The correlations which were $\gamma \geq \pm 0.60$ were considered strongly correlated and a value of $p < 0.05$ was considered statistically significant. For the assessment of haplotype differences, the T-test for independent samples of the same programme was used.

For the assessment of all virulence factors together and with the clinical plus patient-related data as well as for visualisation of relationship, the statistical software CANOCO 5.0 (ter Braak and Smilauer, 2012) was used. The data describing production of virulence factors and clinical and patient-related data were adopted from a previous study performed on the same CAUTI-related strains. For details of the methods and results please see the paper Olejnickova et al. (2014). For the statistical analysis the antibiotics were used alone (amikacin, gentamicin, ceftazidime, cefoperazone, cefepime, cefoperazone/sulbactam, piperacilin/tazobactam, imipenem, meropenem, aztreonam, ciprofloxacin,

ofloxacin, colistin), in groups (*i.e.*, beta-lactams, fluoroquinolones, aminoglycosides and polypeptides), and the total number of all susceptible/resistant antibiotics within the given strain were also calculated. The data, (I) the relationship of pyocins, and (II) the relationship of pyocins with virulence factors production and clinical and patient-related data, were analysed *via* Principal Component Analysis (PCA) and Canonical Correspondence Analysis (CCA), respectively (ter Braak and Smilauer, 2012). These methods are used to seek one or more (mutually independent) gradients applicable as optimal predictors of regression models; if the gradients are found, the models will be capable of expressing the relationships between the data. A detrended correspondence analysis (DCA) was undertaken to check for unimodal distribution of the data (ter Braak and Smilauer, 2012; Lepš and Šmilauer, 2006). In dataset (I), the resulting length of the longest gradient was 3.3; thus, PCA was applied. In dataset (II), 0/1 values of some data led to the choice of a unimodal model, thus the CCA was selected. We ran the PCA with a set of pyocin data to model the response variable; and the CCA with a set of all pyocins, virulence factors and clinical and patient-related data to model the response variable. The statistical significance of the ordination models was tested with Monte-Carlo Permutation Tests assessing the independence of the primary data from the explanatory variables. All the calculated permutation tests reached the level of 499 permutations. The Canoco programme – on the basis of the positive and/or negative values of the tested virulence factors – assessed the rate of their mutual correlation and created the ordination diagram. The weight of the assessment is characterized by the vector size (abscissa) and the tightness of the relationship through the co-sinus of the angle contained in the given vectors. The canonical analyses were calculated with all the relationships together, so the visualisation was more complex than table-presented correlation data; both the canonical analyses ran on three axes, so Figures presented (Figs. 3 & 4) are 2D projections of 3D graphs.

3. Results

3.1. Prevalence of pyocin genes

A collection of 135 CAUTI-related *P. aeruginosa* strains was surveyed for the presence of genes of six S-type pyocins (AP41, S1, S2, S3, S4, and S5), two M-type pyocins (M1 and M4), an L-type pyocin and two tailocin clusters (R- and F- type). The summary results are shown in Fig. 1. A conservative part of the tailocin gene clusters was amplified, their subtypes were not distinguished throughout the study. At least one pyocin gene was detected in 130 strains (96.3 %), the average score was 3.6 pyocin genes per strain (see Fig. 2). A total of 106 isolates harboured at least one genetic element for both soluble and particle type of pyocins. Only soluble pyocin genes were detected in 22 strains (16.3 %) whereas isolates encoding only tailocin determinants were less abundant (2 strains; 1.5 %). In total, soluble pyocin genes were more frequent than the tailocin determinants, the former was presented in 93.6 % of the strains, the latter in 80.0 %. The most common soluble pyocin genes were S4 (76.3 %) and S2 (67.4 %) whilst pyocin L (2.2 %) and M4 (4.4 %) genes were quite rare. An abundance of the R-type pyocin fragment reached 69.6 %, whereas the F-type determinant was presented in 40.0 % of strains. At least one of the S-type pyocins was found in 108 strains (80.0 %), and the M-type in 22 strains (16.3 %).

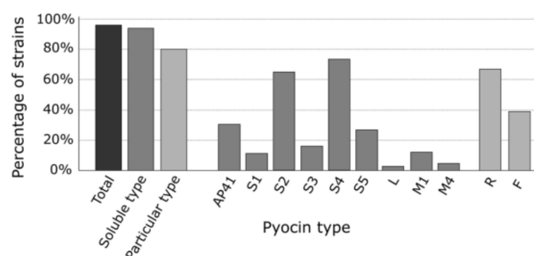

Fig. 1. Bacteriocinogeny and distribution of pyocin determinants in CAUTI-related *P. aeruginosa* isolates.

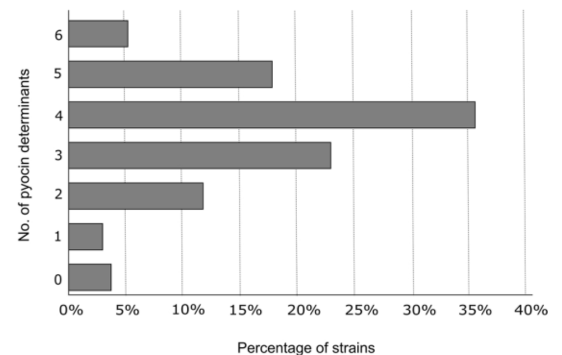

Fig. 2. Prevalence of pyocin determinants.

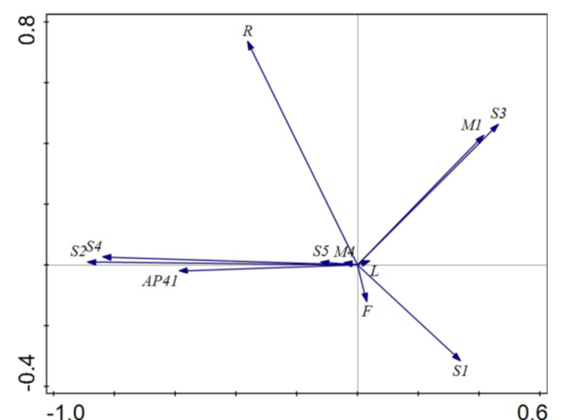

Fig. 3. Principal Component Analysis (PCA) of the relationships among the pyocin genes.
The diagram summarizes patterns of the genes' composition variation across the samples (bacterial strains). Each arrow points in the direction of the steepest increase of the values for the corresponding gene. The angle between the arrows indicates the sign of the correlation between the genes: the approximated correlation is positive when the angle is sharp and negative when the angle is larger than 90 degrees. The length of the arrow is a measure of fit for the given genes.
Key: AP41, S1, S2, S3, S4, S5, L, M1, M4, F, R - Types of pyocins

3.2. Pyocin gene distribution and their co-association with virulence factors

Based on the pyocin genes' occurrence in each strain, 53 producing haplotypes could be distinguished among *P. aeruginosa* strains (see Tab. S2). The most abundant haplotypes harbour genetic elements encoding pyocins AP41, S2, S4, and R (23 strains, 17.0 %), followed by haplotypes S2-S4-S5-F-R (12 strains, 8.9 %) and S2-S4-R-F (8 strains, 5.9 %). On the other hand, in total 34 haplotypes were represented by only one strain.

Analysis of pyocin co-occurrence among CAUTI-related strains showed statistically significant linkages in the pyocin gene distribution (see Table 1 and Fig. 3; for the summary of all statistically significant results and test characteristics see Supplemental Tab. S3). Except for pyocin L, each pyocin type revealed at least one positive and one negative co-association with other pyocins. Positive correlations higher than $\gamma = 0.6$ were detected between 9 pyocin pairs (AP41-S2, AP41-S4,

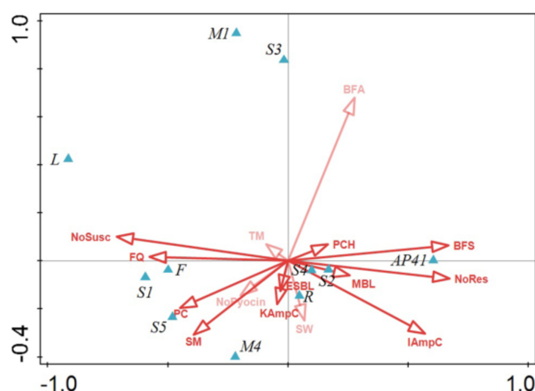

Fig. 4. Canonical Correspondence Analysis (CCA) of the relationships among the pyocin genes and the virulence factors.
The diagram summarizes the variation in the gene composition explained by environmental variables (*e.g.,* sex or age of a patient) and species variables (virulence factors). The gene composition symbols projected perpendicularly onto the line overlaying the arrow of particular environmental/species variables can be used to approximate the optima of individual environmental/species variables in respect to values of that gene composition. The angle between arrows indicates the sign of the correlation between the environmental variables: the approximated correlation is positive when the angle is sharp and negative when the angle is larger than 90 degrees. The length of the arrow is a measure of fit for the given genes. Gene composition symbols: the distance between the symbols approximates the dissimilarity of distribution of relative abundance of those parameters across the samples as measured by their chi-square distance. Points in proximity correspond to genes often occurring together. Only statistically significant cases are visualised.
Key: BFA - Ability to form air-liquid biofilm; BFS - Ability to form submerged biofilm; ESBL - ESBL-producer; FQ – susceptibility to fluoroquinolones; iAmpC - iAmpC-producer; kAmpC - kAmpC-producer; MBL - MBL-producer; NoPyocin – Number of pyocin genes of a strain; NoRes - Number of resistant antibiotics within given strain; NoSusc - Number of susceptible antibiotics within given strain; PC - Pyocyanin producer; PCH – Pyochelin; SM - Swimming motility; SW - Swarming motility; TM - Twitching motility; AP41, S1, S2, S3, S4, S5, L, M1, M4, F, R - Types of pyocins

S1-S5, S1-M4, S2-S4, S3-M1, S5-M4, S5-F, M4-R). Negative associations were more frequent, they were found in 13 cases (AP41-S1, AP41-F, S1-S2, S1-S3, S1-S4, S1-M1, S2-S3, S2-M1, S3-S4, S3-S5, S4-M1, S5-M1, M1-F) in total. For the determination of possible relationships among pyocins and the virulence factors, the relevant data obtained during a previous study (Olejnickova et al., 2014) were added to the dataset. The ability to form submerged biofilm negatively correlated with the harbouring of the pyocin L gene, and swimming motility correlated positively with pyocin M4 (see Fig. 4). Production of siderophore pyochelin was negatively co-associated with the pyocin S1 and S5 genes. Several virulence factors were associated contrarily for pyocins AP41 and L: pyocyanin secretion, overall resistance (total number of resistant antibiotics within the given strain), overall susceptibility, and susceptibility and resistance to fluoroquinolones (see Table 1). Other tested antibiotics and their groups showed no statistically significant correlation with the pyocins production. Overall susceptibility of pyocin AP41 producers (n = 35) was very low; only 11.1 % of strains were sensitive to ciprofloxacin, 16.7 % to ofloxacin, 27.8 % to gentamicin, 33.3 % to cefoperazine and 47.2 % exhibited production of AmpC β-lactamase. The highest effective antibiotics againts AP41 producers were colistin and amikacin (100 % and 97.2 %). Additionally, production of β-lactamases correlated with the presence of several pyocin genes, namely, the positive correlations were: ESBLs-S5; kAmpC-S5; iAmpC-AP41, iAmpC-S2, and the negative correlations were: iAmpC-S1, iAmpC-F, MBLs-F. The statistically significant positive correlation of susceptibility to quinolones was proved for pyocin L and F-type tailocin, whereas the pyocins AP41 and S2 showed a negative correlation (see Table 1 and Supplemental Tab. S3

Table 1
Co-association between pyocin genes (A) and between pyocin genes and virulence factors (B). Coefficient Gama (γ), only correlations of $\gamma > 0.6$ and $\gamma < -0.6$ respectively are listed (N = 135; tested at significance level p = 0.05).

A

Correlation	γ	Z	p-values
AP41 & F	−0.834	−7.193	<<0.001
AP41 & S1	−0.758	−3.212	0.001
AP41 & S2	0.822	6.278	<<0.001
AP41 & S4	0.810	4.150	<<0.001
M1 & F	−1.000	−5.151	<<0.001
M4 & R	1.000	2.451	0.014
S & M1	−1.000	−2.230	0.026
S1 & M4	0.634	2.624	0.009
S1 & S2	−0.833	−6.152	<<0.001
S1 & S3	−1.000	−2.684	0.007
S1 & S4	−0.641	−4.238	<<0.001
S1 & S5	0.663	4.445	<<0.001
S2 & M1	−0.703	−4.867	<<0.001
S2 & S3	−0.722	−5.764	<<0.001
S2 & S4	0.991	13.152	<<0.001
S3 & M1	0.980	12.163	<<0.001
S3 & S4	−0.628	−4.694	<<0.001
S3 & S5	−1.000	−4.665	<<0.001
S4 & M1	−0.686	−4.828	<<0.001
S5 & F	0.711	6.532	<<0.001
S5 & M1	−0.734	−2.992	0.003
S5 & M4	0.707	3.266	0.001

B

Correlation	γ	Z	p-values
AP41 & FQ	−0.742	−6.446	<<0.001
AP41 & NoRes	0.618	6.911	<<0.001
AP41 & NoSusc	−0.623	−6.997	<<0.001
S1 & iAmpC	−1.000	−2.829	0.005
S1 & pyochelin	−1.000	−2.151	0.032
S2 & iAmpC	0.871	4.852	<<0.001
S2 & FQ	−0.320	−2.816	0.005
S4 & iAmpC	1.000	4.459	<<0.001
S5 & pyocyanin	0.546	4.687	<<0.001
S5 & pyochelin	−0.714	−2.829	0.005
S5 & kAmpC	1.000	3.434	0.001
S5 & ESBL	1.000	2.419	0.016
M4 & SM	0.626	3.143	0.002
L & NoRes	−0.768	−2.323	0.02
L & NoSusc	0.776	2.359	0.018
L & FQ	1.000	3.285	0.001
L & BFS	−0.595	−2.137	0.033
F & FQ	0.571	5.681	<<0.001

Key: BFS - Ability to form submerged biofilm; ESBL - ESBL-producer; FQ - Susceptibility to fluoroquinolones; iAmpC - iAmpC-producer; kAmpC - kAmpC-producer; MBL - MBL-producer; NoRes - Number of resistant antibiotics (out of 13 tested antibiotics) within given strain; NoSusc - Number of susceptible antibiotics (out of 13 tested antibiotics) within given strain; SM - Swimming motility; AP41, S1, S2, S3, S4, S5, L, M1, M4, F, R - Types of pyocins.

and Fig. 4). On the other hand, no association of pyocins with production of lytic enzymes (elastase and haemolysins), siderophore pyoverdine, or sex and age of the patient was found.

3.3. *Pyocin gene manifestation and overall competitive interactions among P. aeruginosa strains*

To investigate manifestation of the pyocin genes and their possible contribution to microbial competition in CAUTIs we screened inhibition interactions within a collection of 89 randomly chosen *P. aeruginosa* isolates. From a total of 7656 pairwise assays, 3989 interactions (52.1 %) resulted in inhibition of the recipient strain, in the remaining 47.9 % the recipient strain survived. Each strain was capable of inhibiting a set of other isolates. The number of inhibited strains vary from 24 (27.3 %) to 60 (68.2 %) with an average of 45 (54.5 %) strains. In 43 strains (48.3 %) number of killed strains exceeded the average,16 strains (18.0 %)

were able to inhibit more than 55 strains. On the other hand, only 11 strains (12.4 %) displayed killing activity in less than 35 cases. Each strain was susceptible to at least one antimicrobial agent. The summary data are depicted in Figs. 5 and 6. The observed inhibition interactions were characterized as putative particle or soluble type based on the size of the inhibition zone (France and Remold, 2016; Fyfe et al., 1984). The slightly more frequent was the particle type (*i.e.*, tailocins production), which occurred in 2063 interactions (51.7 % of all inhibitions) whereas 1908 inhibitions (47.8 % of all inhibitions) were mediated by the production of soluble antimicrobials (*i.e.*, S-type, M-type or L-type pyocin). The maximum diameter of the inhibition zone was 13.0 mm (observed in one case). In the case of 13 strains, total of 17 inhibition interactions formed a diameter larger than 10.0 mm. One strain out of these 17 harboured haplotype AP41-S2-S4-R and three strains haplotype S2-S4-S5-R-F. On the other hand, these 17 inhibition interactions were covered by only 9 inhibited partners which seemed to be extraordinarily sensitive to pyocins. Furthermore, we focused on inhibition interactions in the most common haplotypes AP41, S2, S4, R (phenotypically tested 78.3 % of strains as antimicrobials producers) and S2-S4-S5-R-F (tested 83.3 % of strains as antimicrobials producers). Members of both haplotypes inhibited on average less isolates than was average for the collection. A statistically significant reduction was observed for the efficiency of both soluble and particle antimicrobials; for haplotype AP41-S2-S4-R $t = 13.2520$ (soluble inhibition) and $t = 14.3442$ (tailocin-like inhibition); for haplotype S2-S4-S5-R-F $t = 10.8696$ and $t = 10.7497$, respectively, $p << 0.01$ for all statistics. Pairwise inhibition assay included also two mono-pyocin strains. The average diameter of the inhibition zones for the strain encoding pyocin S3 was 3.1 mm ± 0.25 whereas for the strain encoding S4 2 mm ± 0.16.

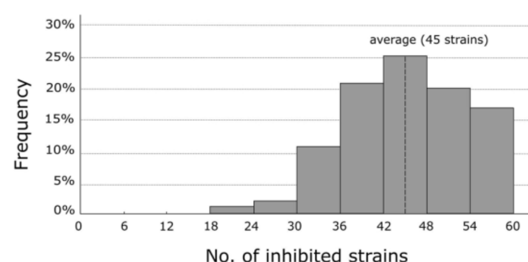

Fig. 6. Histogram showing killing activity of the 89 strains tested in pairwise inhibition assay.

4. Discussion

Pseudomonas aeruginosa is one of the leading causative agents of complicated UTIs which can result in pyelonefritis, urosepsis and death (Mittal et al., 2009). The possible role of pyocin production in urоinfection establishment and maintenance remains unclear despite the fact that bacteriocin production has been described as a putative virulence factor in uropathogenic *E. coli* strains (Azpiroz et al., 2009; Smajs et al., 2010). A few available studies performed on *P. aeruginosa* strains have focused on pyocin production among isolates from cystic fibrosis patients (Bakkal et al., 2010; Bara et al., 2018; Ghoul et al., 2015; Oluyombo et al., 2019) or household isolates (France and Remold, 2016). Our results showed overall high bacteriocinogeny which is in agreement with observations from previous studies. On the other hand, deeper comparison is problematic because: (I) the prevalence of particular pyocin genes varied significantly across the former studies, (II) our inspected collection is much larger than in previously mentioned

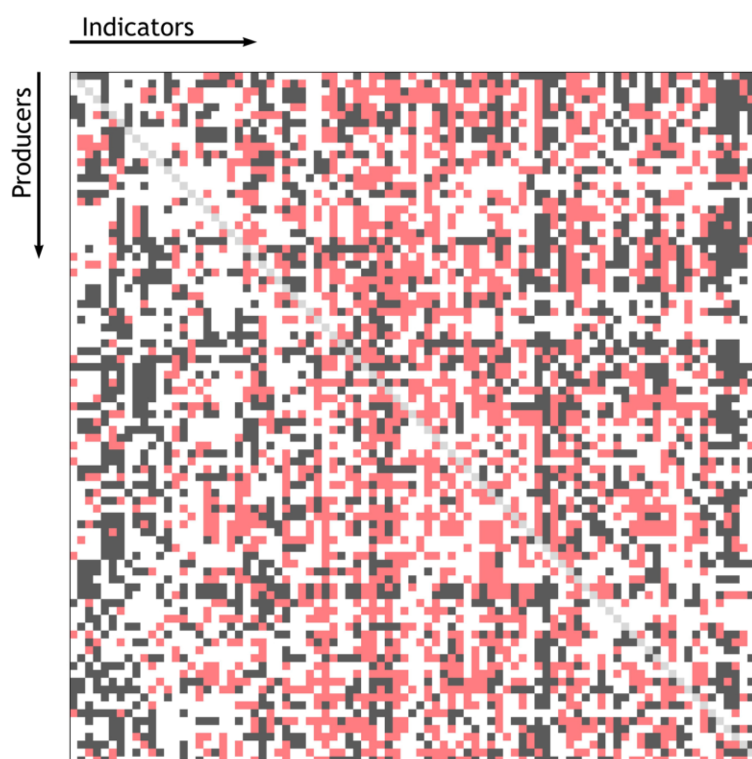

Fig. 5. Overall results of directional killing assay among 89 strains.
Key: Each row represents the killing activity of a single strain, each column showing sensitivity of a single strain. Dark grey boxes indicate inhibition by soluble antimicrobials, red boxes by particle antimicrobials, white boxes showing no inhibition interaction. Light grey boxes symbolize self-on-self tests.

studies working with around 30 isolates per study, (III) pyocins M and L were not assessed in any of the former studies. The pyocins L- and M-type were described predominantly from soil-dwelling and plant-associated pseudomonads (Ghequire et al., 2012; Grinter et al., 2012; Parret et al., 2003), but their production by clinical strains of *P. aeruginosa* was registered previously (Barreteau et al., 2009; McCaughey et al., 2014). This study demonstrated the ability of CAUTI-related *P. aeruginosa* isolates to encode both pyocin types, but their total prevalence was the lowest among all detected pyocins (<5% for pyocin L and M4 and 12 % for pyocin M1) and their ability to inhibit clinical isolates remains unclear because of simultaneous production of several pyocin types. However, we conclude that the average pyocin gene score in CAUTI-related strains is higher than in CF-related isolates (Bara et al., 2018; Ghoul et al., 2015) and household strains (France and Remold, 2016) suggesting the possible role of pyocin production in CAUTIs. A certain role could also be played by the spatial distribution of pyocinogenic strains in the world and extensive research focused on various types of clinical/environmental isolates is needed.

The most frequent genes encoded the soluble pyocins S2 and S4 and tailocin R. All pyocins were involved in the most abundant haplotype covering almost a fifth of the collection and co-occurrence of pyocins S2 and S4 was highly statistically significant. Both toxins use the same pyoverdine receptor FpvAI (Elfarash et al., 2012). Parasitizing on the outer membrane siderophore receptors is a common mechanism how pyocins enter the cells (Denayer et al., 2007; Elfarash et al., 2014, 2012). Pyocin production could play a role in the inter-strain competition in CAUTIs because a low-iron urinary tract environment enhances siderophore production which could boost absorption of pyocins (Cornelis and Dingemans, 2013; Ohkawa et al., 1980). FpvAI is utilized by only S2 and S4 pyocin and the ability to produce pyoverdine was a common feature of the strains (detected in 81.5 % of isolates, data not shown) but neither co-association (positive or negative) between pyoverdine and pyocins was detected. On the other hand, pyochelin, a lower abundant and lower-affinity siderophore (Cornelis, 2010), negatively correlated with pyocin S5 utilising the FptA ferripyochelin receptor (Elfarash et al., 2014). The research of Oluyombo et al. (2019) described that R-tailocin drives competitive interactions in CF-related *P. aeruginosa* biofilms, but in our study the only association of biofilm formation pointed at pyocin L and it was negative. Previous studies have demonstrated that expression of pyocin genes contribute to bacterial susceptibility to quinolones (Brazas and Hancock, 2005; Breidenstein et al., 2008). We detected co-occurrence of fluoroquinolones' susceptibility with pyocin L and F tailocin. On the other hand, we observed negative correlations with pyocins S2 and AP41; the latter was also related to overall antibiotic resistance.

In an attempt to verify expression of the pyocin genes we performed a directional killing assay and compared phenotypic screening of inhibition agent production with pyocin gene detection. Due to the enormous time consumption of the assay, only a random subset of the strains covering two thirds of the possible combinations was tested. Appropriate gene determinants were found for the majority of detected inhibitions, only for three strains we missed a corresponding gene. These mismatches could be explained by a failure in detection of the responsible gene or by the presence of a non-tested determinant. Several new pyocins have been described recently (Dingemans et al., 2016; Naz et al., 2015), which illustrate the insufficient knowledge in this field. On the other hand, one strain with no detected pyocin gene exhibited antagonistic interactions against other isolates. Other antimicrobial compounds described in *Pseudomonas* spp., *e.g.*, type VI secretion effectors or Rhs elements, could be responsible for this observed killing phenotype (Ghequire and De Mot, 2014). Although all obtained results strongly support the connection between the observed killing activity and the presence of the corresponding pyocin gene, a direct crosslink has exceeded the aim of the study. Additionally, we could not link a single pyocin gene and breadth of the inhibited strains because more than one pyocin was harboured in more than 90 % of isolates and tailocin-mediated inhibitions could be hidden by simultaneous production of more diffusible antimicrobials.

Bacteriocins have recently been considered as a potential alternative to antibiotics. Particular pyocins have been shown to exhibit activity against planktonic cells and biofilms (Brown et al., 2012; Redero et al., 2018), inhibition was tested in *in vivo* models (McCaughey et al., 2016) and a synergic effect of pyocins and commercial antibiotics has been described (Smith et al., 2012). During uroinfection treatment, the coating of the catheter surface (Trautner et al., 2005) or bladder irrigation (Riley et al., 2012) by a pyocin cocktail could be beneficial. Several strains obtained during pairwise tests were able to efficiently inhibit biofilm formation of other CAUTI-related strains (data not shown). These promising results will be verified during a subsequent study.

5. Conclusion

Our results demonstrate for the first time the broad inhibition interactions and high prevalence of pyocin genes among CAUTI-related *P. aeruginosa.* The soluble pyocins S4 and S2 and R-type tailocin were the most common across the isolates. Co-occurrence as well as negative association was observed between several pyocin types and between pyocins and virulence factors. In contrast, we were not able to detect any relationship of pyocins with lytic enzymes, siderophore pyoverdine, and patient-related data.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2020.151454.

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