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ANTAGONISTIC EFFECTS OF *PSEUDOMONAS* SPP. ON *ARCOBACTER BUTZLERI*

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Two strains of Arcobacter butzleri (clinical and environmental isolate) were tested for their ability to grow or survive in cell-free supernatant of Pseudomonas sp. All the pseudomonads tested produced antimicrobial substances against A. butzleri in agar diffusion assay (King's B medium). Pseudomonas spp. strains exhibited a broad-spectrum antimicrobial activity against both clinical and environmental A. butzleri strains. The growth inhibition ranged from 20 to 60 % compared with that of control at broth assay. The absorbance of cell-free supernatants was determined and peaks characteristic for siderophores (400 nm) were detected. The addition of iron to laboratory medium partly eliminated the inhibitory activity of all pseudomonas strains. These data indicate the involvement of a siderophore(s) in the antimicrobial activity of pseudomonads against A. butzleri.

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Introduction

In the last decade, gram-negative and rod-shaped bacterium *Arcobacter butzleri* is in a great concern of many scientists. As more sophisticated detection, isolation and identification techniques are developed, more items of information are gathered on their epidemiology and their sources in the environment. Some results previously published proved that some strains could be potentially virulent or toxigenic [1,2]. Thus this species can be ranked to the group of emerging foodborne pathogens. *A. butzleri* was found to be the causative agent in an outbreak in Italian school [3] and was isolated from patients with severe diarrhea [4] and with bacteremia [5]. Water is significant reservoir of *Arcobacter* spp. and may be responsible for their transmission to animals and humans. Recently, *A. butzleri* was isolated from different water sources including river [6], seawater [7], and from surface water [8]. Numerous methods have been developed for successful isolation of arcobacters from environmental sources based mainly on suppressing the accompanying microflora by addition of antibiotics [9,10]. In our recent work, some selective agar plates designed for isolation of *Arcobacter* spp. were allowed to grow other bacteria, among which pseudomonads dominated [8]. These non-fermentative, gram-negative rods were frequently isolated from food and water samples [11] and could accompany *Arcobacter* spp. in their environment. Pseudomonads are known to produce various metabolites including siderophores that sequester available iron and in so doing prevent other microorganisms in the same environment from acquiring sufficient iron for growth.

The objective of this study was to examine the influence of cell-free supernatant, in which *Pseudomonas* sp. were previously grown, on the growth of pathogenic bacterium *Arcobacter butzleri*.

Experimental

Bacteriological media and culture conditions. Six isolates of *Pseudomonas* spp. were used as inhibitory bacteria: UPCE 1 (human isolate), SAL 2 (mayonnaise salad isolate), DEST 1 (distilled water isolate), BAZ 3 (swimming pool isolate), VIR 1 (whirlpool isolate), UMV 1 (isolated from wash basin in hospital). *Ps. fluorescens* CCM 1074 and *Ps. aeruginosa* CCM 3955 were used as reference strains obtained from Czech Collection of Microorganisms, Masaryk University (Brno, Czech Republic). A clinical isolate of *Arcobacter butzleri* CCUG 30484 (Culture Collection, University of Göteborg, Sweden) and environmental *A. butzleri* VODA 1 strain isolated from surface water in our previous study [8] were used as indicator strains. Both inhibitory and indicator bacteria were maintained by monthly transfer at 30 °C on Tryptic Soy Agar slopes (TSA, Merck, Darmstadt,

Germany). All stock cultures were stored at 4 °C. For use in experiment, cultures were transferred twice to King's B broth [12] at 30 °C for 1 day before use.

Detection of inhibitory activity in agar diffusion assay. Antimicrobial activity of substances produced by *Pseudomonas* isolates was determined using modification of procedure described by Cheng [13]. Each inhibitory bacterium was pre-cultured at 30 °C for 1 d, then 5 µl of suspension was pipetted on sterile filter paper disc (12 mm diameter) placed in the center of King's B agar plates. The plates were incubated at 30 °C C for 2 d. After withdrawal of filter paper disc, excess cell growth was removed with a sterile hockey stick, and the plates were exposed to chloroform vapor for 2 h to kill remaining cells. Freshly grown indicator strains adjusting to 10^8-10^9 CFU ml^{-1} (30 °C, 2 d) were surface inoculated onto King's B agar plates that previously contained inhibitory strains and were treated with chloroform. If an inhibitory substance was produced, a zone of no growth appeared around the producer colony after incubation at 30 °C for 2 d.

Siderophore detection in culture supernatant fluids. Two iron-deficient media were used to detect possible siderophore formation. Each pseudomonas strain was separately grown either in 25 ml succinate medium or King's B broth at 30 °C for 2 d at aerobic conditions. After centrifugation (10 000 g, 25 min, 4 °C), the supernatant fluids were adjusted to pH 6.8 with 5 M HCl, then separately passed through 0.2 µm-pore-size membrane filters (Millipore Corporation, Bedford, MA, USA) to remove residual cells. A portion of each supernatant was scanned at 320 to 600 nm against the blank using scanning spectrophotometer (Life Science UV/VIS DU 530, Beckman, USA). King's B broth or succinate medium served as blank in this study. Siderophore-positive cultures peaked at approximately 400 nm, at which the siderophores absorbs. The concentration was calculated using the absorption maximum and the molar absorption coefficient (λ_{max} = 400 nm and ε = 20 000 M^{-1} cm^{-1}) [14].

Testing of antimicrobial activity in broth assay. For the testing of the antimicrobial activity of cell-free supernatant against *A. butzleri*, only King's B broth was used as iron-deficient medium. Four ml of cell free supernatant was dispensed in glass tube and 100 µl of freshly grown indicator strains were separately added yielding initial cell concentration of 10^7-10^8 CFU ml^{-1}. After incubation at 30 °C for 2 d, the growth was monitored at 540 nm. The same procedure was performed with addition of ferric chloride (100 µmol l^{-1}) to supernatant fluid in order to determine whether siderophores contributed to the antimicrobial activity exhibited by *Pseudomonas* spp. isolates. Based on absorbance data at the wavelength of 540 nm, reduction percentage values were calculated to describe the inhibitory effects of the siderophores studied. The absorbance data obtained from monitoring of *A. butzleri* strains growing in King's B broth without siderophore served as control. All the measurements were conducted against pure King's B broth (blank). All experimental procedures were performed in duplicate. If necessary, Student's t-test was applied to detect any significant differences.

Results and Discussion

All the tested pseudomonads formed a visible yellow-green halo with apparent fluorescence at 254 nm (UV lamp) after 2 d incubation at 30 °C in King's agar plates. Two reference stains and six isolates of *Pseudomonas* spp. exhibited a broad-spectrum antimicrobial activity against both *A. butzleri* CCUG 30484 and *A. butzleri* VODA 1 (Table I).

Table I Inhibitor production by various *Pseudomonas* species on King's B agar plates

Inhibition by	Indicator strain of *A. butzleri*	
	CCUG 30484	VODA 1
Ps. fluorescences CCM 1074	++	++
Ps. aeruginosa CCM 3955	+	+
Pseudomonas spp.		
UPCE 1	++	+
SAL 2	+++	++
DEST 1	+++	+++
BAZ 3	+	+
VIR 1	++	+
UMV 1	++	+

\+ zone of inhibition < diameter of producer colony
++ zone of inhibition = diameter of producer colony
+++ zone of inhibition > diameter of producer colony

Chemical substances of only two *Pseudomonas* spp. (DEST 1 and SAL 2) isolates produced in iron-deficient agar medium clearly inhibited the growth of both *A. butzleri* strains, i.e. no growth was observed in yellow-green halo and the inhibition zones were clearly terminated. The rest of inhibitory strains also inhibited the growth of *A. butzleri* with colony forming even in yellow-green halo in some cases. When cell-free supernatant was used in the experiment, however, our results did not correlate with those obtained in agar assay. For example, *Pseudomonas* spp. isolated from swimming pool (BAZ 3) showed a little inhibitory effect on the growth of *A. butzleri* strains in agar plate assay, while in liquid medium the pronounced growth inhibition was observed. This could be partly explained by the production of brown pigment into King's B liquid medium, which was also visually detected, and could be responsible for enhanced antimicrobial effect. Brown color formation could be caused either by non-

enzymatic autooxidation of catechol-like substances [15] or from transforming of tyramine [16]. The formation of brown pigment was also observed after growing of *P. syringae* isolates in King's B medium [17]. However, our data do not allow us to specifically implicate metabolite(s) in the active mechanism of inhibition. In contrast to our findings, the inhibitory action was not detected when both target and test cultures were grown in liquid media [18]. The growth of *A. butzleri* CCUG 30484 was mainly inhibited by the antimicrobial substances of four *Pseudomonas* spp. (DEST1, BAZ3, SAL2 and UPCE1) strains produced in low-iron medium. The growth inhibition was 50-60 % compared with that of the control. Significant differences in growth inhibition were observed between clinical and environmental isolates of *A. butzleri* in iron-deprived liquid medium. Generally, the clinical isolate of *A. butzleri* (CCUG 30484) was more sensitive to antimicrobial substances of *Pseudomonas* spp. than the environmental isolate (VODA1). It is well known that environmental bacterial strains are more resistant than clinical strains to antimicrobial agents [19]. Addition of iron to the substrate eliminated the inhibitory activity of all the inhibitory strains (Figs 1 and 2).

The absorbance of cell-free supernatants was determined and peaks characteristic of siderophores (400 nm) were detected in all cell-free supernatants obtained after appropriate incubation of pseudomonas strains. Siderophores are characterized as low molecular mass, diffusible, water soluble, UV-fluorescent (yellow-green), Fe(III)-specific ligands [14]. They have been recognized as po-

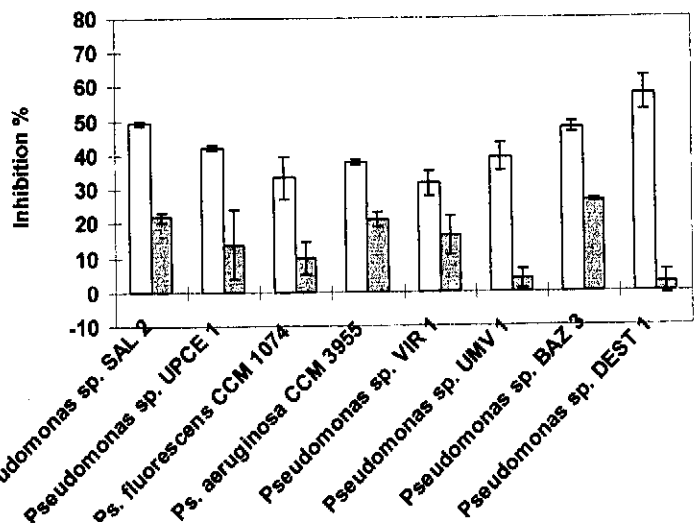

Fig. 1 Effect of culture supernatant fluids of *Pseudomonas* spp. on the growth of *Arcobacter butzleri* CCUG 30484 in King's B medium without (open bars) or with 100 μmol l^{-1} iron added (closed bars). Bars represent standard errors of means

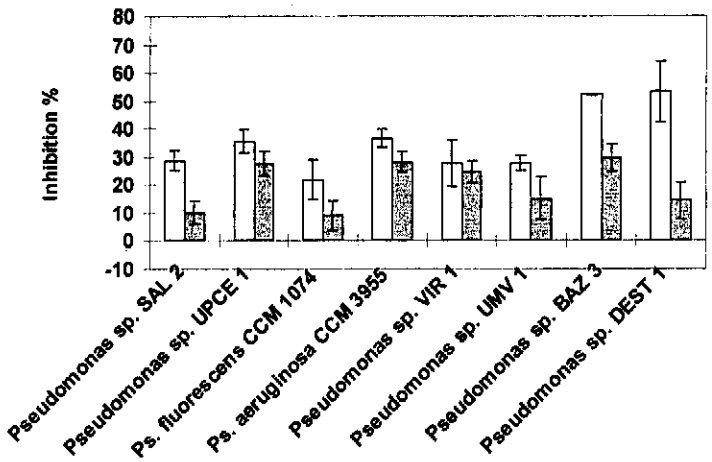

Fig. 2 Effect of culture supernatant fluids of *Pseudomonas* spp. on the growth of *Arcobacter butzleri* VODA 1 in King's B medium without (open bars) or with 100 μmol l^{-1} iron added (closed bars). Bars represent standard errors of means

tent inhibitors of some pathogenic bacteria [13,18,20,21]. Despite the fact that *Arcobacter* spp. is a closely related species to *Campylobacter* spp., it does not necessarily mean that the control treatments are sufficient in the same way as for campylobacters. For example, *A. butzleri* is more tolerant than *C. jejuni* to irradiation treatment [22]. The recent literature documents both the antimicrobial activity of siderophores against *Campylobacter jejuni* [13] and utilization of exogenous siderophores produced by other microorganisms as iron carriers [23,24]. In this study, sterile-filtered supernatant of *Pseudomonas* spp. strains showed bacteriostatic effect on the growth of *A. butzleri* strains. A complete growth inhibition of *A. Butzleri* after exposure to EDTA has been described previously [25], but a higher concentration of chelating agent was used in this study. Strong anti-*Shewanella* activity was established due to siderophore [18], whereas Freedman [20] did not attribute antimicrobial activity to siderophores. It is also apparent from our results that the antimicrobial activity of inhibitory strains was not completely eliminated by iron supplementation in this study. Thus their activity may be attributable to the production of several different bacterial inhibitors such as HCN [26] or bacteriocin-like substances [27,28]. Two isolates (SAL 2 and DEST 1) attained the highest concentration of siderophores in both iron-deficient media tested, whereas with *Ps. fluorescens* CCM 1074 the lowest siderophore concentration was observed (Table II.).

The growth inhibitory effects did not show correlation with the siderophores concentration observed in low-iron liquid media. The pH of the King's B broth increased a little bit from 7.2 to 7.6 in average after the growth period, and to pH 8.0 in the case of the use of succinate medium.

Table II Concentration of siderophores produced in two laboratory media measured at 400 nm

Pseudomonas isolate	King's B broth [a]μmol l^{-1}	Succinate medium [a]μmol l^{-1}
Ps. fluorescences CCM 1074	5.4	5.0
Ps. aeruginosa CCM 3955	6.2	24.0
Pseudomonas spp.		
UPCE 1	8.9	19.7
SAL 2	15.2	21.1
DEST 1	16.8	22.5
BAZ 3[b]	12.6	20.1
VIR 1	12.1	21.5
UMV 1	10.3	18.6

[a] calculated using absorption maximum and the molar absorption coefficient (λ_{max} = 400 nm and ε = 20 000 M^{-1} cm^{-1})[14]
[b] contained brown pigment

In conclusion, these data indicate the involvement of a siderophore(s) in the antimicrobial activity of pseudomonads against *A. butzleri*. Further investigations are necessary to establish the production of iron chelating substances by *Arcobacter* spp. and thus elucidate its behavior in iron deprived conditions with relation to its pathogenicity. This is probably the first report describing bacterial antagonism of *Pseudomonas* spp. against newly emerged pathogenic bacteria *A. butzleri*.

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