Effects of Ampicillin and Vancomycin on *Staphylococcus aureus* Biofilms

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Abstract

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The collection of 23 coagulase-positive *Staphylococcus aureus* strains isolated mainly from food in the Czech Republic were tested on the ability to form biofilms in the presence of ampicillin and vancomycin. The antimicrobial sensitivity (16 antibiotics) was determined in all strains by the standard disc diffusion method on Mueller-Hinton agar plates (NCCLS). The resistance to ampicillin was found in 16 strains (69.5%), all strains being susceptible to vancomycin. The formation of biofilm was conducted in 96-well, polystyrene microtiter plates COSTAR 3797 in tryptic soy broth (TSB) with 1% of glucose for 24 h at 30°C. Staining with crystal violet (0.1%) was used for biofilm quantification. Ampicillin (0.5, 2, and 4 mg/l) and vancomycin (32, 64 and 128 mg/l) were added: (*i*) direct addition of the agent to the well at zero time, (*ii*) after 24 h to washed well, (*iii*) after 24 h directly to well with the cell suspension. The tested types of ampicillin treatment did not confirm the impact of resistance on the biofilm production among the strains tested. The addition of vancomycin at zero time of cultivation effectively suppressed the biofilm production. Other types of treatment showed unequal strain dependent response. Planktonic cells demonstrated a higher sensitivity to antibiotics than the biofilm forming cells.

Keywords: *Staphylococcus aureus* strain; planktonic cells; ampicillin; vancomycin

Staphylococcus aureus is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, biofilm forming capacity and antibiotic resistance. The organism colonises the nasal passages, skin and mucous membranes of humans, and it is also found on the skin and hair of warm-blooded animals. Its capacity to produce human diseases has not diminished with the introduction of antibiotics (Chambers 2001). *S. aureus* can alter its genotype and/or phenotype to adapt to its surroundings. One of the defense mechanisms of *S. aureus* is the capacity to form biofilms that provides the cells living in this arrangement 10 to 1000 – fold higher antibiotic resistance in comparison with planktonic cells (Stewart & Costerton 2001). The presence of coagulase-positive staphylococci in food is regulated by EU Directive No. 1441/2007 and their numbers ought to be under control.

In natural habitats, the biofilm formation is an important survival strategy for bacteria that can be embedded within a self-produced extracellular polymeric matrix of polysaccharides, proteins, lipids, and nucleic acids (Donlan & Costerton 2002; Flemming & Wingender 2010). Biofilms are also involved in the pathogenesis of various infections related to implanted medical devices and staphylococci are among the most frequent etiological agents. Bacterial biofilms have received continued attention in the food-processing industry due to their increased resistance towards desiccation, heat, acids, preservatives, disinfectants, and antibiotics (Chmielewski & Frank 2003; Shi & Zhu 2009; Van Houdt & Michiels 2010; Purkrtová *et al*. 2011). Many bacteria co-ordinate the expression of genes essential for virulence and survival by the production, secretion, and detection of small signal molecules in a process termed "quorum sensing". Autoinducer-2 (AI-2), considered to be a universal language for interspecies communication, is synthesised by the

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LuxS enzyme (Vendeville *et al*. 2005). Ahmed *et al*. (2007) demonstrated a relation between the inactivation of luxS manifested in reduced biofilm formation and increased susceptibility to erythromycin and ampicillin.

The properties of biofilms resulting in their increased resistance to antibiotics are not thoroughly understood. The ability to hold up the inhibitory effects of antibiotics is attributed to two main mechanisms, i.e. the biofilm architecture that prevents the antibacterial agent from reaching its target by electrostatic repulsion or sequestration by surface polymers (Otto 2008), or slowing down this process by limited diffusion or repulsion (Xu *et al*. 1996). Ampicillin, a broad-spectrum aminopenicillin, is one of the most widely used antibacterial drugs in veterinary medicine. The exposure to antibiotics often results in further selection of homologous resistant strains, a phenomenon mainly favoured by irrational antibiotic administration. Since the introduction of ampicillin, many bacteria have developed resistance to penicillins owing to the production of the β-lactamases (Prescott & Baggot 1993). As a solution of this event, new types of antimicrobials agents were introduced. Vancomycin is the most reliable therapeutic agent against infections caused by β-lactamase armed bacteria like meticillin-resistant *S. aureus* (MRSA). The mechanisms of vancomycin resistence are still poorly understood. A large number of genes, including regulators and encoding proteins functioning in the cell wall metabolism, are upregulated in vancomycin-intermediate *S. aureus* (VISA) strains (Gardete *et al*. 2006).

Therefore, our concern was to find whether the varying susceptibility to 16 antimicrobial drugs affects the formation of biofilms in the presence of ampicillin and vancomycin. For this reason, we investigated the biofilm formation of 23 strains of coagulase-positive *S. aureus* with various levels of antibiotics resistance (22 food isolates, 1 clinical) from the Czech Republic under the influence of ampicillin and vancomycin using *in vitro* models (microtiter plates). The objective of the experiments was (*i*) to compare ampicillin and vancomycin effects on the biofilm formation and planktonic cells viability, (*ii*) to measure the biofilm cells viability under different ways of antibiotics application (directly on biofilm or in the suspension).

MATERIAL AND METHODS

Culture preparation. All the 23 strains of *S. aureus* isolated from different food matrices were obtained from the National Institute of Public Health, Prague, Czech Republic, and are shown in Table 1. Stock cultures were stored at –80°C in brain-heart infusion (BHI; Merck, Darmstadt, Germany) and 15% glycerol. The working cultures were maintained on Baird-Parker agar plates (BHI; Merck, Darmstadt, Germany) at 4°C for 30 days. Prior to each experiment, one colony from Baird-Parker agar was grown in 6 ml of BHI at 37°C for 24 hours.

Examination of sensitivity to antibiotics. All 23 strains of *S. aureus* were tested for their sensitivity to 16 antimicrobial compounds by standard disc diffusion method (DDM) according to NCCLS (2002). The results are summarised in Table 2.

Biofilm cultivation in microtiter plate. Microtiter polystyrene plates COSTAR 3797 (Corning Inc., Lowell, USA) were used as standard tools in

Table 1. List of the tested *S. aureus* strains obtained from National Institute of Public Health, Brno, Czech Republic. Enterotoxin encoding genes were detected by PCR method

Strain No.	Sample origin	Type of SEs
672	patisserie	A, H
673	patisserie	D, I, G
711	balkan salt cheese	H
719	chicken tetrazzini	E
720	faeces	A, C, E
740	pork ham	C
816	sea fish	A, B
817	spinach	C
921	raw cow milk	D, I, G, J
940	meat product mass	A
992	pork ham	B
1003	long-life salami	B, D, J
1041	raw cow milk	D, J
1106	patisserie	B
1117	patisserie	C, I, G
1141	dry sausage	C
1173	pickled Camembert	A, B, D, J
1176	chopped raw meat	D, J
1185	sea fish	A
1238	cow milk	–
1241	cow milk	–
1247	poultry salami	A, C, I, G
1249	raw cow milk	B, I, G

SE – staphylococcal enterotoxine; SEs – plural

Table 2. Resistance of the *S. aureus* strains to tested antibiotics (oxacillin, tetracycline, erytromycin, chloramphenicol, ampicillin, penicillin, gentamicin, ciprofloxacin, vancomycin, teicoplanin, rifampin, methicillin, cefoperazone, amoxycillin/clavulanic acid, trimetoprim/sulfomethoxazole, quinupristin/dalfopristin)

Resistance pattern	Number of strains	Strain number
Sensitive to 16 tested antibiotics	7	672, 711, 740, 816, 817, 992, 1106
AMP^R	4	673, 719, 1141, 1185
AMP^R, PEN^R	5	720, 940, 1041, 1173, 1249
AMP^R, PEN^R, OXA^R	2	1003, 1176
AMP^R, PEN^R, OXA^I	2	1241, 1247
AMP^R, PEN^R, OXA^I, CFP^I	1	1117
AMP^R, PEN^R, CFP^I	1	1238
AMP^R, PEN^R, RIF^R, ERY^I	1	921

TET – tetracycline; CMP – chloramphenicol; AMP – ampicillin; PEN – penicillin; OXA – oxacillin; CFP – cefoperazone; RIF – rifampin; ERY – erytromycin; ^{R}resistant; ^{I}intermediate

all experiments. Overnight cultures grown in BHI were diluted in tryptone-soya broth with 1% of glucose – TSBG (Merck, Darmstadt, Germany) and equilibrated at 0.5 McFarland value (app. 10^8 CFU). The microtiter plates wells were filled with 100 µl of individual strain culture and incubated at 30°C for 24 hours. This procedure was followed by crystal violet staining according to the protocol designed by Djordevic *et al*. 2002, and adapted by Purkrtová *et al*. (2011). Gram-positive bacteria like *S. aureus* have a thick mesh-like cell wall made of peptidoglycan (50–90% of cell envelope), which is stained purple by crystal violet. Crystal violet is a basic dye binding to negatively charged surface molecules, including live and dead bacteria and matrix polysaccharides.

Measurement of planktonic cells growth. The concentrations of bacterial suspensions growing in microtiter plate wells were measured at 620 nm by spectrophotometer Tecan-Spectra 9440012 (Tecan Ltd., Grödig, Austria). Five ampicillin and vancomycin treatment assay.

Three following concentrations of ampicillin were tested: 0.5, 2, and 4 mg/l. These concentrations were made up in 100 µl wells of the microtiter plate. For vancomycin, the final concentrations were 32, 64, and 128 mg/l. A range of layouts were used for the measurements of planktonic cells and biofilms.

*Layout No. 1***.** Effect of 24 h antibiotic presence on both planktonic cells growth and biofilm formation. To the microtiter plates wells containing 100 µl of cell suspension the tested concentrations of ampicillin and vancomycin were added. After 24 h of incubation, optical density of the bacterial suspension (planktonic growth) was measured first in the wells, and after pouring out the contents the biofilms created on the walls of the wells were stained with crystal violet and evaluated.

Layout No. 2. Direct antibiotics treatment effects on biofilm cells. The cell suspensions after 24 h incubation were removed from the wells and the microtiter plate wells were washed 6 times with 350 µl of sterile distilled water to remove the loosely associated bacteria and were left to dry. The tested antibiotics in the volume of 100 µl were added directly onto the biofilm for 10 min treatment. After this time, the wells were washed 6 times with 350 µl of sterile distilled water and 100 µl of fresh sterile TSBG was added to each well.

Layout No. 3. Indirect antibiotics treatment effects on biofilm cells.100 µl of each antibiotic tested was added directly into the 24 h grown suspension in the wells and after 10 min the plates were washed out and fresh TSBG was added as described in No. 2.

Layout No. 4. Before washing, as described in No. 3, 10 µl of the treated suspension was transferred into a new well with 90 µl of TSBG to test the antibiotics treatment effect on planktonic cells.

Statistics. Each concentration and positive control (without antibiotics) was measured in four parallel wells using two independent assays for each strain and antibiotic. The obtained values were statistically evaluated by using the software Statistica 8 (StatSoft, Inc., Tulsa, USA). The values lying in 70% lower percentile were taken in account for the determination of the average and standard deviation. The interval determined by the average and the double standard deviations (visualised in graphs as the error bars) consists of 95% of the considered values.

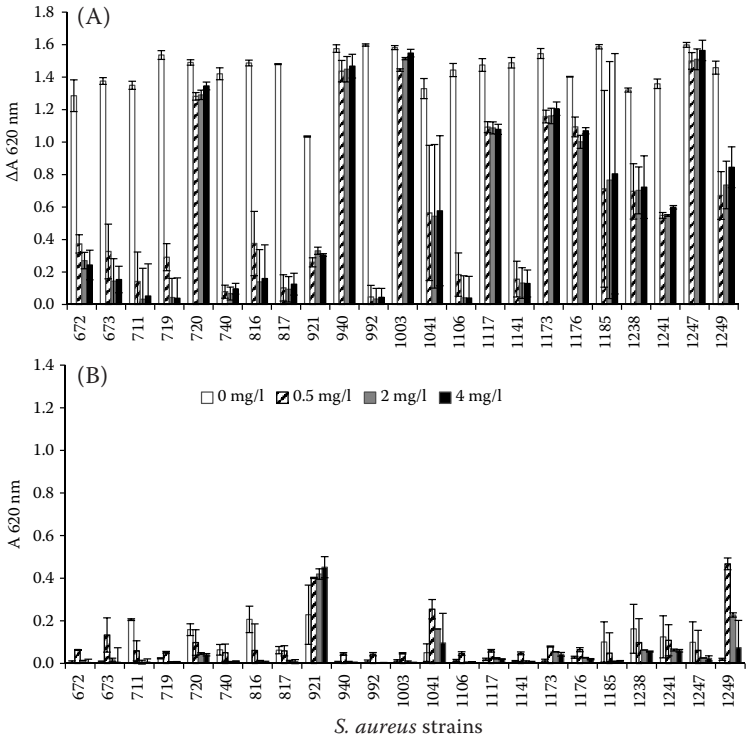

Figure 1A. The ability of *S. aureus* planktonic cells to grow in ampicillin presence for 24 hours

OD 620 nm correspond to cell density

Figure 1B. The formation of *S. aureus* biofilm in ampicillin presence for 24 hours

The biofilm formation represented by crystal violet staining, measured at OD 620 nm

RESULTS

According to the results of DDM of ampicillin and the planktonic cell growth (Table 2), it is possible to divide the tested *S. aureus* strains in the presence of this agent into several groups while the vancomycin response was uniform, all strains being susceptible.

The first group includes seven strains (720, 940, 1003, 1117, 1173, 1176, 1249) that were found resistant of DDM, were growing even at the highest ampicillin concentration (4 mg/l) in the same manner as the control cells, the reduction of the growth being maximally 20% (Figure 1A). Three strains (627, 719, 1141) showing resistence by DDM were suppressed in growth by

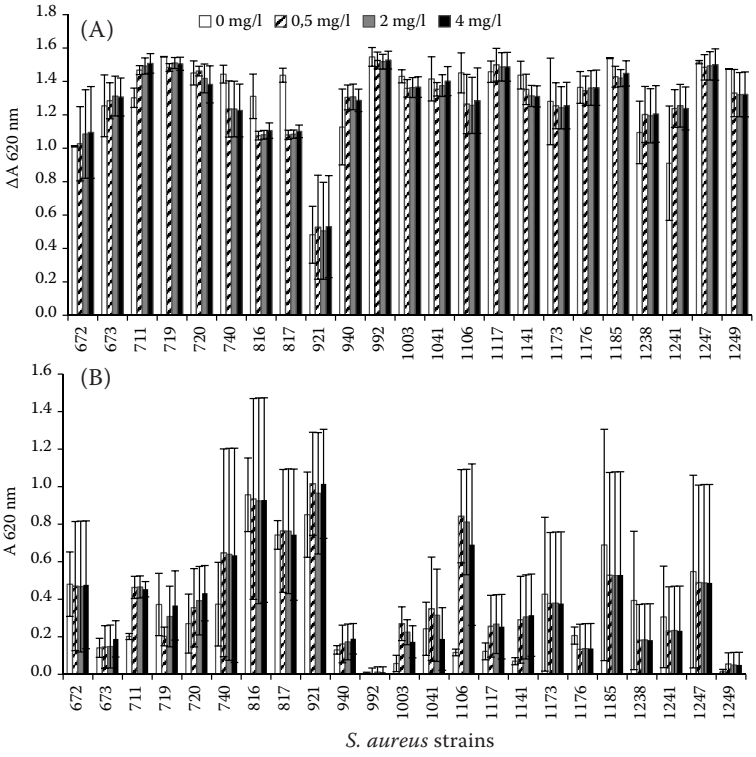

Figure 2A. The ability of *S. aureus* biofilm cells treated 10 min by ampicillin to grow *S. aureus* strains

Procedure: To microtitre plates after 24 h cultivation of *Staphylococcus aureus* were added various concentrations of ampicillin for 10 min. After this time the microtiter plates were washed and fresh TSBG was added. Values in graph were measured after subsequent 24 h cultivation. OD 620 nm corresponds to cell density

Figure 2B. The biofilm formation by *S. aureus* biofilm cells treated for 10 min with ampicillin

The biofilm formation represente by crystal violet staining, measured at OD 620 nm

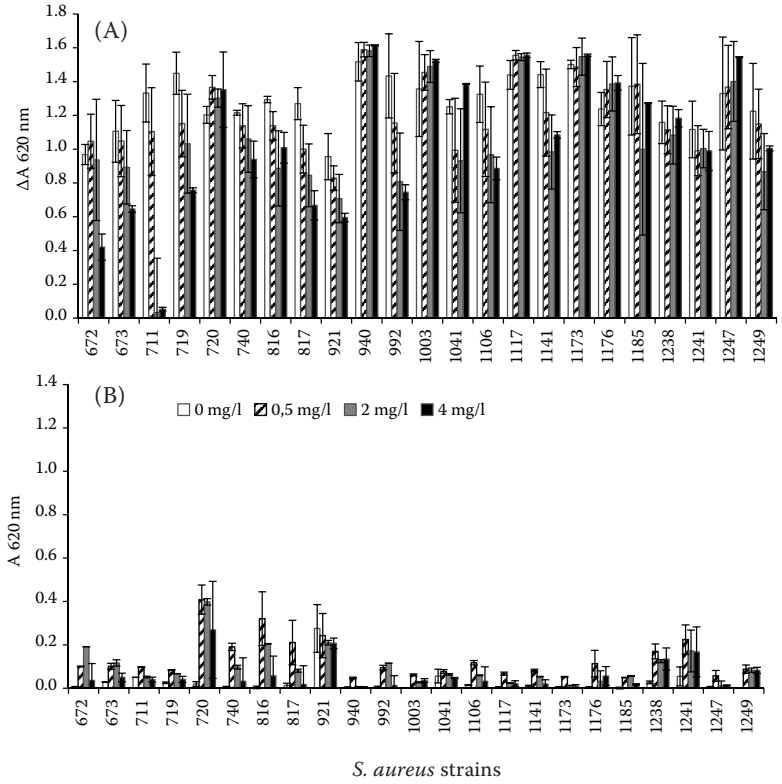

Figure 3A. The ability of *S. aureus* planktonic cells treated for 10 min with ampicillin to grow

Procedure: To microtitre plates after 24 h cultivation of *Staphylococcus aureus* were added various concentrations of ampicillin for 10 min treatment, 10 µl of this cell suspension served as inoculum of new microtiter wells filled with 90 µl of TSBG. OD 620 nm corresponds to cell density

Figure 3B. The biofilm formation of *S. aureus* planktonic cells treated 10 min with ampicillin

Procedure: To emptied microtiter plates after 24 h cultivation of *Staphylococcus aureus* were added various concentrations of ampicillin for 10 min treatment. After washing fresh TSBG was added. Values in graph were measured by crystal violet staining after 24 h cultivation at OD 620 nm

80% using ampicillin concentration of 0.5 mg/l. Next five strains (921, 1041, 1238, 1241, and 1249) were also shown resistant by DDM, but the reduction in growth was only 50%. The effect of ampicillin on the biofilm formation given in Figure 1B was also differentiated. The group of three strain (921, 1041, and 1249) resistant to ampicillin was induced to the biofilm formation by 0.5 mg/l. Ampicillin concentration of 0.5 mg/l induced the biofilm formation by 14 strains in total while it simultaneously suppressed the planktonic cell growth.

The biofilm formation by strain 921 (ampicillin resistant) showed the dependence on ampicillin

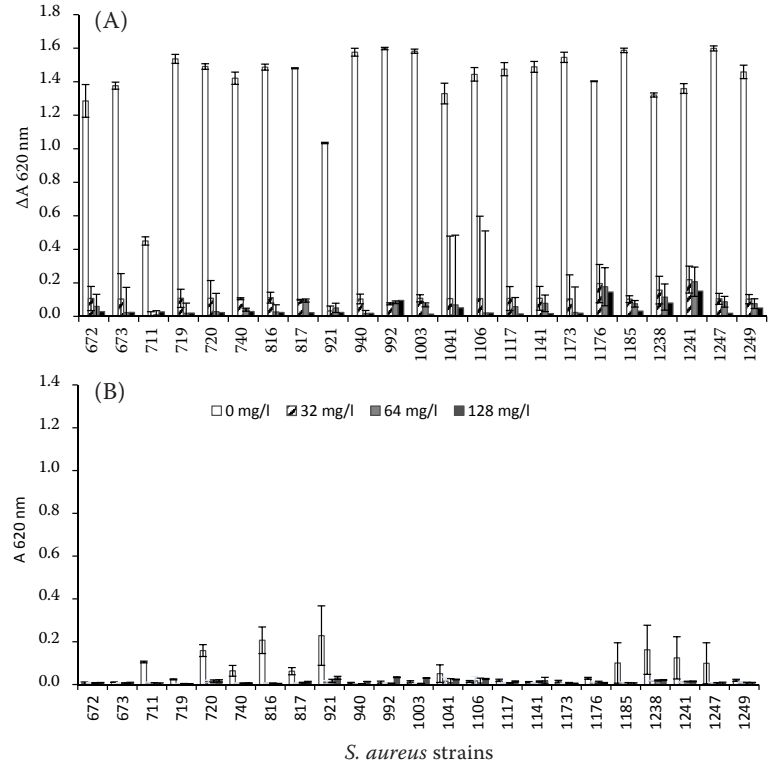

Figure 4A. The ability of *S. aureus* planktonic cells to grow in vancomycin presence for 24 hours

OD 620 nm corresponds to cell density

Figure 4B. The formation of *S. aureus* biofilm in vancomycin presence for 24 hours

The biofilm formation represented by crystal violet staining, measured at OD 620 nm

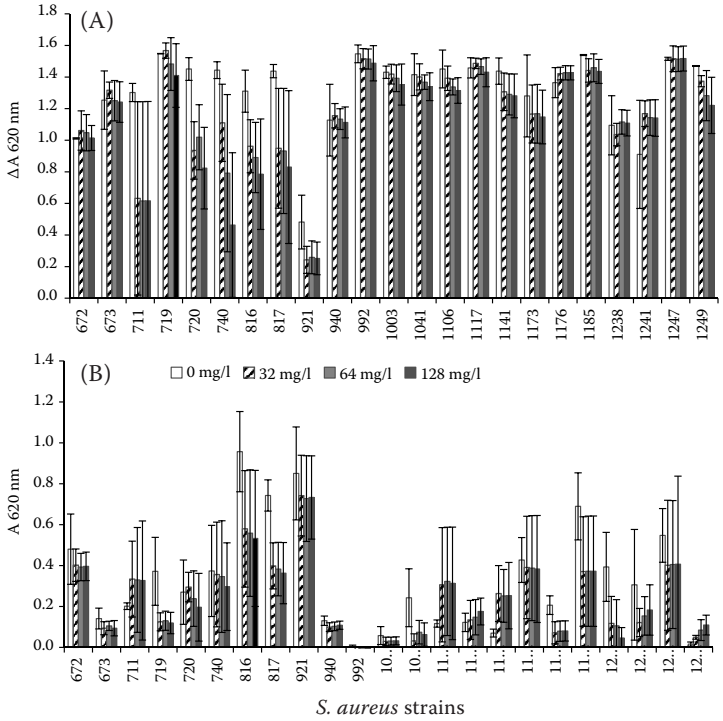

Figure 5A. The ability of S. aureus biofilm cells treated 10 min with vancomycin to grow

Procedure: To microtitre plates after 24 h cultivation of *Staphylococcus aureus* were added various concentrations of vancomycin for 10 min treatment. After this time the microtiter plates were washed and added fresh TSBG was added. Values in graph were measured after subsequent 24 h cultivation. OD 620 nm corresponds to cell density

Figure 5B. The biofilm formation of *S. aureus* biofilm cells treated 10 min with vancomycin

The biofilm formation of *Staphylococcus aureus* after 24 h by 10 min vancomycin treatment

concentration. Similar behaviour was also observed in other strains (740, 1041, 1249 etc.).

A short (10 min) treatment of 24 h old suspension by ampicillin resulted in good planktonic cell growth after following 24 h (Figure 2A). A similar treatment (10 min) followed by cultivation in fresh TSB did not stop the biofilm formation (Figure 2B). The effect of ampicillin was strain dependent: in 10 strains, the biofilm formation was induced, 10 others were not substantially influenced, and in the rest the biofilm formation was reduced by 40% to 80%. In Figure 3A are summarised the results of planktonic growth after 10 min ampicillin treatment directly on the biofilm, the wells being filled with fresh TSB. The formation of biofilm by the cells treated with ampicillin for10 min for10 min is shown in Figure 3B.

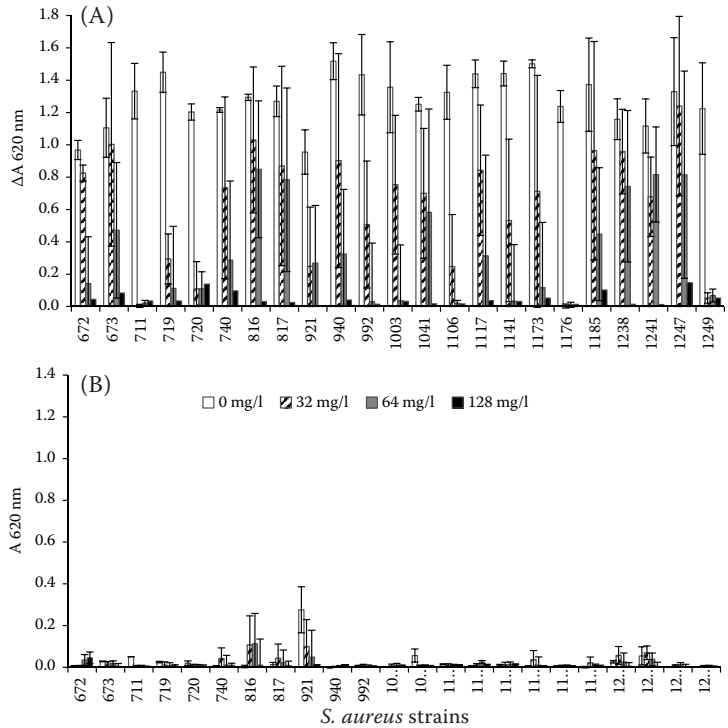

Figure 6A. The ability of *S. aureus* planktonic cells treated 10 min by vancomycin to grow

Procedure: To microtitre plates after 24 h cultivation of *Staphylococcus aureus* were added various concentrations of vancomycin for 10 min treatment. 10 µl of this cell suspension served as inoculum for new microtiter wells filled with 90 µl of TSBG. OD 620 nm corresponds to cell density

Figure 6B. The biofilm formation of *S. aureus* planktonic cells treated 10 min with vancomycin

Procedure: To emptied microtiter plates after 24 h cultivation of *Staphylococcus aureus* were added various concentrations of vancomycin for 10 min treatment. After washing fresh TSBG was added. Values in graph were measured by crystal aviolet staining after 24 h cultivation at OD 620 nm

The antibiotic effect showed a similar trend as in the previous layout.

The effect of 24 h treatment vancomycin on the biofilm formation and planktonic cells growth was unambiguous. In Figure 4, a strong inhibition is documented of both the biofilm formation and planktonic cells growth. The 10 min treatment with various vancomycin concentrations reduced the subsequent biofilm formation in a strain dependent manner (Figure 5). Figure 6A shows the planktonic growth after 10 min treatment with vancomycin in the suspension and Figure 6B shows the biofilm formation in wells inoculated with cells treated in this way.

The behaviour of strain No. 921 was peculiar because this strain grew in comparable conditions better in the form of biofilm than as planktonic cells.

DISCUSSION

The biofilm development is an important component of bacterial survival. In the study presented here, we demonstrated that all 23 tested coagulase-positive *S. aureus* strains were able to adhere and most of them were able to produce biofilms at a significant level. It means that *S. aureus* strains existing outside the clinical environments, in our case in foods, also possess this capacity. The effects of antimicrobial agents on the formation of biofilm were preferably studied with strains colonising various types of catheters or implantates (Ceri *et al*. 1999; Berrington & Gould 2001). Currently, there is no universally accepted *in vitro* model of the biofilm development, treatment, and quantification, while microtiter plates of polystyrene are employed quite often (Djordjevic *et al*. 2002; Peeters *et al*. 2008; Croes *et al*. 2009). Unfortunately, there is no universally accepted method for studying the antibiotic susceptibility of bacteria in biofilms. One of the first studies published by Yarwood *et al*. (2004) found that the ability to form biofilms is connected with the protection against antibiotics. Using two cell-wall-active antimicrobial agents, the susceptibility of *S. aureus* tested as planktonic cells was compared to the susceptibility of the biofilm cells cultivated on microtiter plates. Our results did not produce unambiguous answer on the relationship between the antibiotic resistance and capacity to form biofilm,

The impact of various concentrations of ampicillin and vancomycin on the quantity of biofilm revealed great differences between the antimicrobial efficacy of the agents tested. Evidence exists that subinhibitory concentrations of some antibiotics promote the biofilm formation (Hoffman *et al*. 2005), perhaps by facilitating the extracellular polymeric substance production. In our experiments such a behaviour was exhibited by strain 921. The collection of *S. aureus* strains the studied was rather heterogeneous as to the origin, production of enterotoxines, and antibiotic resistance patterns. In our collection (23 strains) only seven strains showed sensitivity to ampicillin. It could be only anticipated that the exchange of strains between human and animal hosts is rather extensive, because ampicillin is used only in human medicine. Further research has to be conducted into *S. aureus biofilms* and antibiotics resistance under different parameters. While a prolonged presence of the antibiotics tested reduced the biofilm formation, a short treatment of an established biofilm (10 min) with either antibiotics tested was not so efficient.

To the best of our knowledge, it is evident that quorum-sensing is the principal controlling mechanism of the biofilm formation. The *S. aureus* quorum-sensing system is encoded by the accessory gene regulator (*agr*) locus and the responsible molecule that it produces and senses is called an autoinducing peptide (Ji *et al*. 1997; Boles & Hoeswill 2008). For a better understanding of the differences between the capacities to form biofilm, the genetic background of the strains followed ought to be investigated.

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