ORIGINAL PAPERS

Adv Clin Exp Med 2007, **16**, 5, 619–628
ISSN 1230-025X

Elżbieta Litwinek[1], Jerzy Piątkowski[2], Izabela Dolna[1], Beata Kowalska-Krochmal[1], Anna Przondo-Mordarska[1]

Tetracycline Resistance Mechanisms of *Staphylococcus Aureus* Strains Isolated from Patients in Wrocław*

Mechanizmy oporności na tetracyklinę u szczepów *Staphylococcus aureus* izolowanych od chorych z terenu Wrocławia

[1] Department of Microbiology, Silesian Piasts University of Medicine in Wrocław, Poland
[2] Institute of Genetics and Microbiology, University of Wrocław, Poland

Abstract

Background. The mechanism of the antibacterial activity of tetracyclines consists in protein biosynthesis inhibition. Resistance to these drugs is caused either by modification of tetracycline's ribosomal target site or by the activity of efflux pumps which extrude the antibiotic from the bacterial cell. Reserpine is one of the known efflux pump inhibitors. It is unknown to what extent the efflux mechanism determines tetracycline resistance among the *S. aureu*s strains isolated from patients in Wrocław. The aim of this study was to identify transporters which remove tetracycline from *S. aureus* cells.
Material and Methods. The level of tetracycline resistance was determined in *S. aureus* by serial antibiotic dilutions in solid media and by estimating MIC according to NCCLS guidelines. The tetracycline resistance genes *tet(K), tet(L)*, and *tet(M)*, *tet(O)* were detected by PCR. Tetracycline accumulation in cells was measured using a radioactively labeled form of it by a method described elsewhere.
Results. Of the tetracycline efflux pump genes, only *tet(K)* was identified. The second resistance determinant with regard to the frequency of occurrence was *tet(M)*, wchich is responsible for a tetracycline target side modification In the strains with the efflux system, application of a pump inhibitor caused an increase in tetracycline accumulation in the cell. In contrast, the tetracycline cell concentration was decreased in the strains which did not possess the efflux mechanism.
Conclusions. In the patient-derived strains of *S. aureus*, efflux-dependent tetracycline resistance determined by the Tet(K) transporter was found to predominate. In the strains with a high level of tetracycline resistance, the transporter coexisted with Tet(M*)* protein, responsible for antibiotic target-site modification. A study using a radioisotopic method indicated that another, as yet unknown pump for tetracycline removal exists in *S. aureus* cells (**Adv Clin Exp Med 2007, 16, 5, 619–628**).

Key words: antibiotic resistance, tetracycline, membrane transporters*, tet(K)*, *tet(M).*

Streszczenie

Wprowadzenie. Mechanizm przeciwbakteryjnego działania tetracyklin polega na inhibicji biosyntezy białka. Oporność na tę grupę antybiotyków u *S. aureus* jest wywołana modyfikacją miejsca ich docelowego działania w rybosomie lub aktywnością pomp wyrzucających antybiotyk na zewnątrz komórki. Inhibitorem pomp jest m.in. rezerpina. Nie ustalono, jak często mechanizm aktywnego wypływu decyduje o oporności na tetracykliny u szczepów *Staphylococcus aureus* izolowanych od chorych z Wrocławia.
Cel pracy. Identyfikacja pomp usuwających tetracyklinę z komórek *S. aureus*.
Materiał i metody. Poziom oporności *S. aureus* na tetracyklinę określono metodą seryjnych rozcieńczeń antybiotyku w podłożu stałym, ustalając wartość MIC antybiotyku wg zaleceń NCCLS. Obecność genów *tet(K)*, *tet(L)* oraz *tet(M)*, *tet(O)* stwierdzono za pomocą techniki PCR. Wewnątrzkomórkową akumulację tetracykliny mierzono z użyciem znakowanego izotopowo antybiotyku wg metody opisanej przez Wondrack et al.

* The paper was financed by Silesian Piasts University of Medicine in Wroclaw, grant no. 1218.

Wyniki. Spośród genów kodujących pompy dla tetracykliny, zidentyfikowano tylko *tet(K)*. Drugą, co do częstości występowania, determinantą genetyczną był gen *tet(M)*, odpowiadający za zmianę miejsca docelowego działania antybiotyku. U szczepów posiadających mechanizm aktywnego wypływu (*efflux*) zastosowanie inhibitora pomp powodowało wzrost kumulacji tetracykliny w komórkach, a u szczepów pozbawionych tego mechanizmu obserwowano zmniejszanie wewnątrzkomórkowego stężenia antybiotyku.
Wnioski. U szczepów *S. aureus* izolowanych z terenu Wrocławia dominującym mechanizmem oporności na tetracykliny jest aktywny wypływ antybiotyku zależny od transportera Tet(K). U szczepów wysoko opornych współwystępuje on z białkami Tet(M) modyfikującymi miejsce docelowego działania tej grupy antybiotyków. Badania izotopowe wskazują również na obecność w komórkach badanych szczepów innego nieznanego dotąd transportera usuwającego tetracykliny (**Adv Clin Exp Med 2007, 16, 5, 619–628**).

Słowa kluczowe: antybiotykooporność, tetracyklina, transportery błonowe, *tet(K)*, *tet(M).*

Tetracyclines comprise a group of bacteriostatic antibiotics which act as protein biosynthesis inhibitors by blocking aminoacyl-tRNA binding to a ribosomal acceptor site [1, 2]. Their broad antibacterial spectrum of action, involving both Gram-positive and Gram-negative bacteria, as well as the relatively low cost of therapy have been reasons for the excessive application of tetracyclines not only in humans, but also in animals and plants [1, 2]. A common negative consequence of the ubiquitous existence of sub-inhibitive concentrations of these antibiotics in our environment currently observed is a decrease in their therapeutic efficiency. This is because some microorganisms have developed mechanisms of resistance to these compounds as a result of strong selective pressure and horizontal gene transfer, the primary source for which were probably *Streptomyces* as antibiotic bio-producers [2].

Staphylococcus aureus (SA) is a pathogenic species to humans which exhibits a high percentage of tetracycline-resistant strains. This applies especially to methicillin-resistant SA strains (MRSA), in which a lack of tetracycline sensitivity has been detected in over 75% of isolates, and to methicillin-sensitive strains (MSSA) isolated from both ambulatory and hospital patients among whom the resistance frequency attained no less than 15% [1].

So far, two main tetracycline resistance mechanisms in *Staphylococcus aureus* have been described, each determined by the occurrence of mobile genetic elements such as plasmids and transposons [3]. The first is the efflux mechanism, which consists in active export of tetracycline from the cell with the assistance of the transporting proteins Tet(K) or Tet(L) located in the plasma membrane. These proteins, coded by the genes *tet(K)* and *tet(L)*, respectively, belong to the MFS transporter superfamily. Acting as a proton pump, they exchange the antibiotic/Mg^{+2} complexes in the cytoplasm for H^+ ions (antiport), which leads to a decrease in the intracellular tetracycline concentration and thus makes it difficult for the antibiotic to attain the target sites located in ribosomes [4–6]. The second mechanism governing tetracycline bacterial resistance is due to the ribosomal protection proteins Tet(M) and Tet(O), coded by the *tet(M)* and *tet(O)* genes, respectively. Attachment of either of these proteins to a ribosome disables the antibiotic from attaching, thus preventing it from efficient protein biosynthesis inhibition. Both resistance mechanisms exhibit the inductive mode of action [2, 7].

The plant alkaloid reserpine as well as the compound carbonyl cyanide m-chlorophenyl hydrazone (CCCP) both restore the sensitivity of *S. aureus* to tetracycline if the resistance is determined by the efflux proteins coded by the *tet(K)* or *tet(L)* genes [8, 9]. Unfortunately, these compounds are not applied in therapy because of their toxicity to the human organism. However, they are useful in *in vitro* research for determining to what extent the observed bacterial resistance is caused by the efflux mechanism [1].

The application of efflux inhibitors is justifiable when the epidemiological situation regarding the resistance mechanisms in the population of tetracycline-resistant strains is known. Unfortunately, data about how frequently the efflux mechanism occurs among *S. aureus* strains have so far been very sparse in Poland. The aim of the study presented here was to test a series of tetracycline-resistant *S. aureus* strains collected from hospital and ambulatory patients and to determine whether the efflux mechanism may be a significant cause of their resistance.

Material and Methods

One hundred twenty-nine strains were isolated as resistant or intermediate resistant to tetracycline from different materials (sputum, urine, upper and lower airway swabs, ear swabs, conjunctival sac swabs, wound swabs, genitourinary tract swabs) taken from patients from 2003 to 2004. The resistance level of *S. aureus* was determined by serial antibiotic dilutions in Mueller Hinton agar and MIC (minimum inhibitory concentration) estima-

tion according to the NCCLS guidelines [10]. The following criteria for tetracycline were used: MIC ≥ 16 μg/ml indicated a resistant strain, MIC = 8 μg/ml an intermediate-resistant strain, and and MIC ≤ 4 μg/ml a tetracycline-sensitive strain. The final MIC value was estimated on the basis of at least three measurements.

To identify strains with respect to resistance caused by the efflux pump, reserpine at a concentration of 20 μg/ml was applied as the pump inhibitor in media containing the antibiotic. The sensitive wild strains *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used as a quality control for the antimicrobial susceptibility testing.

The genetic tetracycline resistance determinants *tet(K)*, *tet(L)*, *tet(M)*, and *tet(O)* were detected using PCR. DNA was prepared as follows: three to five colonies from overnight culture on blood agar plates were suspended in 40 μl of distilled water and boiled for 10 minutes. After centrifugation (13,000 rpm, 40 sec), the supernatant was applied as the DNA template (2.5 μl/reaction). Amplification was performed in the reaction mixture with a final volume of 50 μl according to the formula described previously [11] with modifications using Tag PCR Core Kit (Qiagen). The respective primers, reaction conditions, and expected amplification product lengths are listed in Table 1. The initial denaturation was performed at 94°C for 3 min and the final elongation at 72°C for 5 min. The obtained products were separated, as shown in Fig. 1, in 1.2% agarose gel with ethidium bromide using the O'Range Ruler 200-bp DNA Ladder (Fermentas) as a molecular-weight marker.

Tetracycline efflux was measured by monitoring the antibiotic uptake into the log-phase *S. aureus* cells according to the formula described by Wondrack et al. [12]. The formula was modified in that after 30 min of antibiotic uptake, the reacting mixture was divided into two parts to which CCCP or reserpine was added at final concentrations of 10 μg/ml and 20 μg/ml, respectively. The experiments were performed independently with three concentrations of radiolabeled tetracycline: 0.015 μg/ml, 0.044 μg/ml, and 0.15 μg/ml. The antibiotic, with a radioactivity of 1 Ci/mmol, was obtained from Perkin Elmer LAS, Inc. The radioactivity levels of filtered cells were counted by a Wallac 1409 Liquid Scintillation Counter and shown in CPMs (counts per minute).

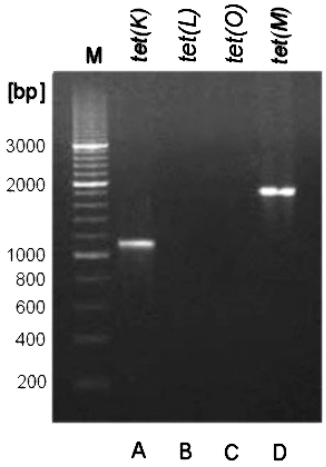

Fig. 1. Electrophoretic separation of PCR products for an *S. aureus* strain in which two tetracycline resistance determinants, *tet(K)* and *tet(M)*, were detected. M – molecular-weight marker. Lanes A–D: *S. aureus* 43

Ryc. 1. Rozdział elektroforetyczny produktów reakcji PCR dla szczepu *S. aureus,* u którego wykryto dwie determinanty oporności na tetracyklinę *tet(K)* i *tet(M)*. M – marker wielkości. Ścieżki A–D: *S. aureus* 43

Results

The *S. aureus* strains collected from microbiological laboratories in Wroclaw were tested for

Table 1. Primers and PCR conditions used to detect tetracycline resistance determinants

Tabela 1. Startery i warunki reakcji PCR zastosowane do wykrycia determinant oporności na tetracyklinę

Target gene (Gen docelowy)	Primer sequences (Sekwencje startowe)	No. of PCR cycles (Liczba cykli PCR)	PCR conditions (Warunki reakcji PCR)	PCR fragment size (Wielkość produktu PCR)
tet(K)	5' – TATTTTGGCT TTGTATTCTT TCAT – 3' 5' – GCTATACCTG TTCCCTCTGA TAA – 3'	35	1 min at 95°C, 1 min at 50°C, 1 min 30 s at 72°C	1159 bp
tet(L)	5' – ATAAATTGTT TCGGGTCGGT AAT – 3' 5' – AACCAGCCAA CTAATGACAA TGAT – 3'	35	1 min at 95°C, 1 min at 50°C, 1 min 30 s at 72°C	1077 bp
tet(M)	5' – AGTTTTAGCT CATGTTGATG – 3' 5' – TCCGACTATT TAGACGACGG – 3'	35	1 min at 95°C, 1 min at 50°C, 1 min 30 s at 72°C	1862 bp
tet(O)	5' – AGCGTCAAAG GGGAATCACT ATCC – 3' 5' – CGGCGGGGTT GGCAAATA – 3'	35	1 min at 95°C, 1 min at 55°C, 1 min 30 s at 72°C	1723 bp

Table 2. The level of tetracycline resistance in *S. aureus* strains

Tabela 2. Poziom oporności na tetracyklinę u szczepów *S. aureus*

Resistance phenotype (Fenotyp oporności)	MIC (μg/ml)[1)]	No (%) of strains (Liczba (%) szczepów)
R*	256	2 (1.5)
	128	13 (10.1)
	64	22 (17.0)
	32	78 (60.5)
	16	13 (10.1)
I*	8	1 (0.8)

[1)] MIC – minimal inhibitory concentration.
* according to the NCCLS guidelines: R – resistant (MIC ≥ 16 μg/ml), I – intermediate (MIC = 8 μg/ml), S – susceptible (MIC ≤ 4 μg/ml).

[1)] MIC – minimalne stężenie hamujące antybiotyku.
* Według reguł NCCLS: R – oporny (MIC ≥ 16 μg/ml), I – zmniejszona wrażliwość (MIC = 8 μg/ml), S – wrażliwy (MIC ≤ 4 μg/ml).

their sensitivity to tetracycline and classified into several classes with respect to their resistance levels according to the scale described above. For the tetracycline-resistant strains, MIC values within the range of 16–128 μg/ml were obtained. Only one strain exhibited a lowered MIC value and was classified as an intermediate-resistant one. For over 60% of the strains the MIC value was 32 μg/ml and for approximately 30% it was no less than 64 μg/ml, as shown in Table 2.

The resistance experiment in the presence of reserpine allowed distinguishing strains which could be expected to have an efflux pump. This compound appeared to influence the tetracycline resistance levels of particular strains to different extents, which enabled classifying them into groups according to the criteria of: 1) the MIC obtained without reserpine and 2) the level of sensitivity restored by it (Table 3). The most numerous group (102 strains) comprised strains in which reserpine caused restoration of full sensitivity to tetracycline. Among these strains, the Tet(K) pump was identified as the only resistance determinant. A much smaller group of 22 strains was resistant and their MIC values were not modified by reserpine or modified very slightly (by factors of 2–4). These resistance phenotypes were caused by either the coexistence of more than one genetic determinant governing different resistance mechanisms or a mechanism connected only with the *tet(M)* gene coding for the ribosome-protecting protein. The changes in MIC 50 values by reserpine for particular classes of strains grouped according to their resistance levels show that the more times this compound decreases the resistance level, the lower the final MIC obtained.

Table 3. Influence of reserpine on tetracycline resistance of *S. aureus* strains

Tabela 3. Wpływ rezerpiny na tetracyklinooporność szczepów *S. aureus*

Phenotype (Fenotyp)		MIC 50 (μg/ml)[3]		MIC decrease (Zmniejszenie MIC)	No. (%) of strains (Liczba (%) szczepów)		Resistance determinants (Determinanta oporności)
T[1]	T + res[2]	T	T + res				
R*	S*	32	0.125	256 ×	4	(3.1)	*tet(K)*
		64	0.5	128 ×	9	(6.9)	*tet(K)*
		32	0.5	64 ×	22	(17.0)	*tet(K)*
		32	1	32 ×	54	(41.9)	*tet(K)*
		16	1	16 ×	12	(9.3)	*tet(K)*
I*	S	8	1	8 ×	1	(0.8)	*tet(K)*
R	R	256	64	4 ×	1	(0.8)	*tet(K), tet(M)*
		128	64	2 ×	13	(10.0)	*tet(K), tet(M)*
		32	32	–	1	(0.8)	*tet(K), tet(M)*
		32	32	–	7	(5.4)	*tet(M)*

[1)] Tetracycline.
[2)] Reserpine.
[3)] Minimal inhibitory concentration for ≥ 50% of strains.
* According to the NCCLS guidelines: R – resistant (MIC ≥ 16 μg/ml), I – intermediate (MIC = 8 μg/ml), S – sensitive (MIC ≤ 4 μg/ml).

[1)] Tetracyklina.
[2)] Rezerpina.
[3)] Minimalne stężenie hamujące dla ≥ 50% szczepów.
* Wg rekomendacji NCCLS: R – oporny (MIC ≥ 16 μg/ml), I – zmniejszona wrażliwość (MIC = 8 μg/ml), S – wrażliwy (MIC ≤ 4 μg/ml).

Tabela 4. Nieoczekiwana oporność na tetracyklinę u szczepów *S. aureus*

Table 4. Unexpected modes of resistance to tetracycline in *S. aureus* strains

Phenotype (Fenotyp)		MIC (μg/ml)[3]		MIC decrease (Zmniejszenie MIC)	No. (%) of strains (Liczba (%) szczepów)		Resistance determinants (Determinanta oporności)
T[1]	T + res[2]	T	T + res				
R*	S*	16	0.5	32 ×	1	(0.8)	not detected
		32	4	8 ×	1	(0.8)	not detected
R	I*	64	8	8 ×	1	(0.8)	*tet(K)*
R	R	64	32	2 ×	1	(0.8)	*tet(M)*
		128	64	2 ×	1	(0.8)	*tet(K)*

[1)] Tetracycline.
[2)] Reserpine.
[3)] Because of the small number of strains, MIC 50 was not determined.
* According to the NCCLS guidelines: R – resistant (MIC ≥ 16 μg/ml), I – intermediate (MIC = 8 μg/ml), S – sensitive (MIC ≤ 4 μg/ml).

[1)] Tetracyklina.
[2)] Rezerpina.
[3)] Ze względu na małą liczbę szczepów nie oznaczono wartości MIC 50.
* Wg rekomendacji NCCLS: R – oporny (MIC ≥ 16 μg/ml), I – zmniejszona wrażliwość (MIC = 8 μg/ml), S – wrażliwy (MIC ≤ 4 μg/ml).

The phenotypes of five strains presented in Table 4 were unexpected. Two of the strains became susceptible to tetracycline in the presence of reserpine (MIC decreases by factors of 8 and 32) although no known genetic resistance determinant was recognized in those strains. Two other strains lacked tetracycline susceptibility in the presence of reserpine in spite of a detected Tet(K) pump and a decrease in the MIC value (by factors of 2 or 8).

The genetic analysis revealed that the majority of the tetracycline-resistant strains (> 90%) which became more sensitive when reserpine was applied possessed an efflux pump-determining gene *tet(K)*, which codes for the transporter protein Tet(K). There was no other known tetracycline efflux pump gene identified in the tested strains. Tet(K) coexisted with Tet(M) in almost 12% of strains. This refers mainly to the strains having the highest resistance levels (MIC 128–256 μg/ml). The *tet(L)* and *tet(O)* genes were not identified in any of the strains tested. In 6.2% of the strains only *tet(M)* occurred.

The results shown in Table 1 seem to provide satisfactory evidence for the existence of the tetracycline efflux mechanism in the collected strains of *S. aureus.* Direct evidence was obtained with the assistance of the radioisotopic method. In the first stage of experiments, three strains, i.e. 1) sensitive to tetracycline, 2) resistant to this antibiotic and possessing the efflux pump, and 3) resistant but lacking this pump, were tested for their cellular ability to accumulate the radioactively labeled antibiotic in the presence or absence of reserpine. The first was the wild type *S. aureus* ATCC 25923 strain, the second the tetracycline-resistant *S. aureus* 133 strain in which *tet(K)* had been identified, and the third the tetracycline-resistant *S. aureus* 105 strain lacking the pump. An antibiotic concentration of 0.15 μg/ml was used in the reaction mixtures. The results of the tests are shown in Figure 2.

As can be inferred from the results in Figure 2a, the antibiotic accumulated in the wild-type strain in the absence of reserpine. A similar effect was observed in the resistant strain not possessing the efflux pump (Fig. 2c). This effect, however, was lowered by reserpine in both strains. An opposite effect was observed for the resistant strain (Fig. 2b) in which the pump is present: the intracellular antibiotic concentration did not rise within 10–40 min of incubation, although an increase was observed after 40 min. Reserpine stimulated the process of accumulation, unlike in the two other strains.

The inhibitory influence of reserpine on the tetracycline accumulation by the wild-type strain was intriguing. It was also interesting to determine why the antibiotic concentration rose significantly after 40 min of incubation. The question arises as to whether the extracellular concentration of the antibiotic is responsible for the rapid increase in its intracellular concentration. To answer this, experiments were performed in which the radioactively labeled tetracycline concentration was reduced to 0.044 μg/ml and another efflux pump inhibitor, CCCP, was applied instead of reserpine. It was interesting to find out if CCCP would exhibit a

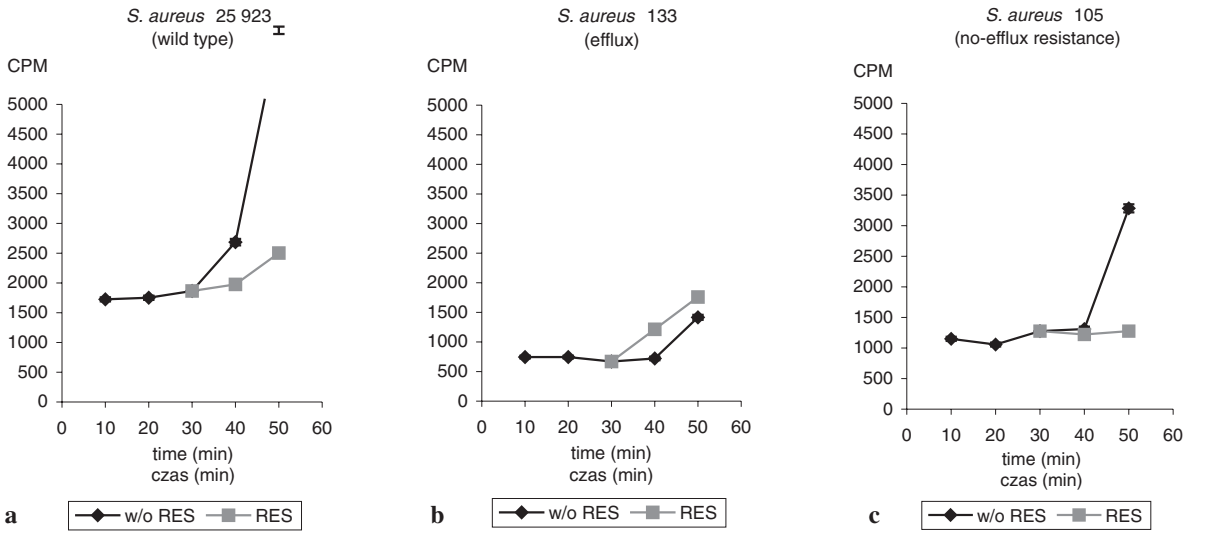

Fig. 2. Accumulation of tetracycline in cells of *S. aureus* strains in the presence of reserpine: **a** – wild-type, sensitive to tetracycline, lacking the Tet(K) efflux pump; **b** – resistant, possessing the efflux pump; **c** – resistant, not possessing the efflux pump – another mechanism of resistance: Tet(M). The antibiotic concentration in the reaction mixture was 0.15 μg/ml

Ryc. 2. Akumulacja tetracykliny przez komórki *S. aureus* w obecności rezerpiny: **a** – typ dziki, wrażliwy na tetracyklinę, niemający transportera Tet(K); **b** – oporny, mający pompę Tet(K) usuwającą ten antybiotyk; **c** – oporny, niemający tej pompy – inny mechanizm oporności: Tet(M). Antybiotyk w mieszaninie reakcyjnej występował w stężeniu 0,15 μg/ml

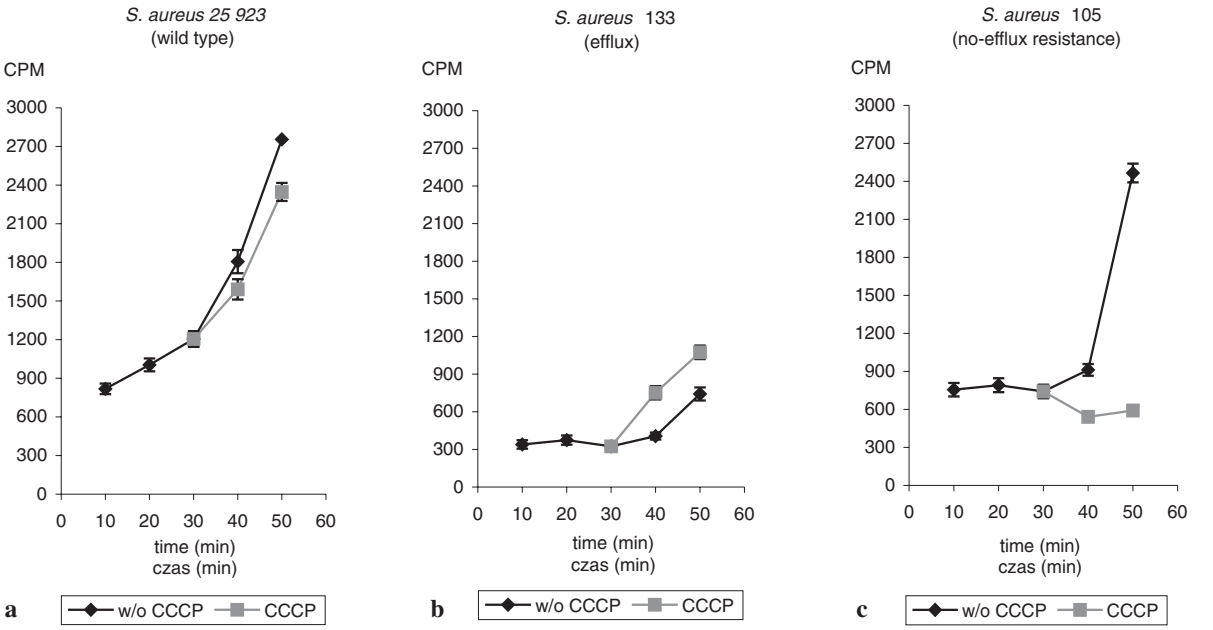

Fig. 3. Accumulation of tetracycline in cells of *S. aureus* strains in the presence of CCCP: **a** – wild-type, sensitive to tetracycline, lacking the Tet(K) efflux pump; **b** – resistant, possessing the efflux pump; **c** – resistant, not possessing the efflux pump – another resistance mechanism: Tet(M). The antibiotic concentration in the reaction mixture was 0.044 μg/ml

Ryc. 3. Akumulacja tetracykliny przez komórki *S. aureus* w obecności CCCP: **a** – typ dziki, wrażliwy na tetracyklinę, niemający transportera Tet(K); **b** – oporny, mający pompę usuwającą ten antybiotyk; **c** – oporny, niemający tej pompy – inny typ oporności: – Tet(M). Antybiotyk w mieszaninie reakcyjnej występował w stężeniu 0,044 μg/ml

similar mode of inhibitory/stimulatory action as reserpine. Furthermore, if CCCP turned out to be able to identify the efflux system as effectively as reserpine, it should be more useful to consider the obtained results because the molecular mechanism governing CCCP's action is much better known than that of reserpine. The results of these experiments are shown in Figure 3.

These results indicate that CCCP's effect regarding antibiotic accumulation is almost identical to that of reserpine. This compound reduced the tetracycline concentration inside the cells both in the wild-type strain (Fig. 3a) and in the resistant one with no efflux pump (Fig. 3c). As for the resistant strain with an active efflux system, CCCP significantly increased the antibiotic concentration inside the cells (Fig. 3b).

Even if a lower antibiotic concentration was applied, the antibiotic concentration inside the cells rapidly increased after 40 min of incubation. That is why the question arises whether this is only an extracellular tetracycline concentration-dependent effect or, perhaps, some other complex mechanism governs this enhanced accumulation. To answer this question, final supporting tests were performed using five strains differing in tetracycline resistance and efflux pump possession. The extra-cellular antibiotic concentration lowered again, this time to 0.015 μg/ml (Figs. 4 a–e).

As shown by the results gathered in Fig. 4, stimulation of antibiotic accumulation by CCCP occurred in each strain in which there was a Tet(K) efflux pump system (Figs. 4b, 4c). The strains lacking the efflux system reacted to CCCP in such a way that the antibiotic accumulation inside the cells significantly decreased (Figs. 4a, 4d, 4e). As for the range of accumulation after 40 min, a higher increase in antibiotic concentration was noted in strain SA 150 (Fig. 4e), which had earlier been identified as resistant but lacking the efflux pump.

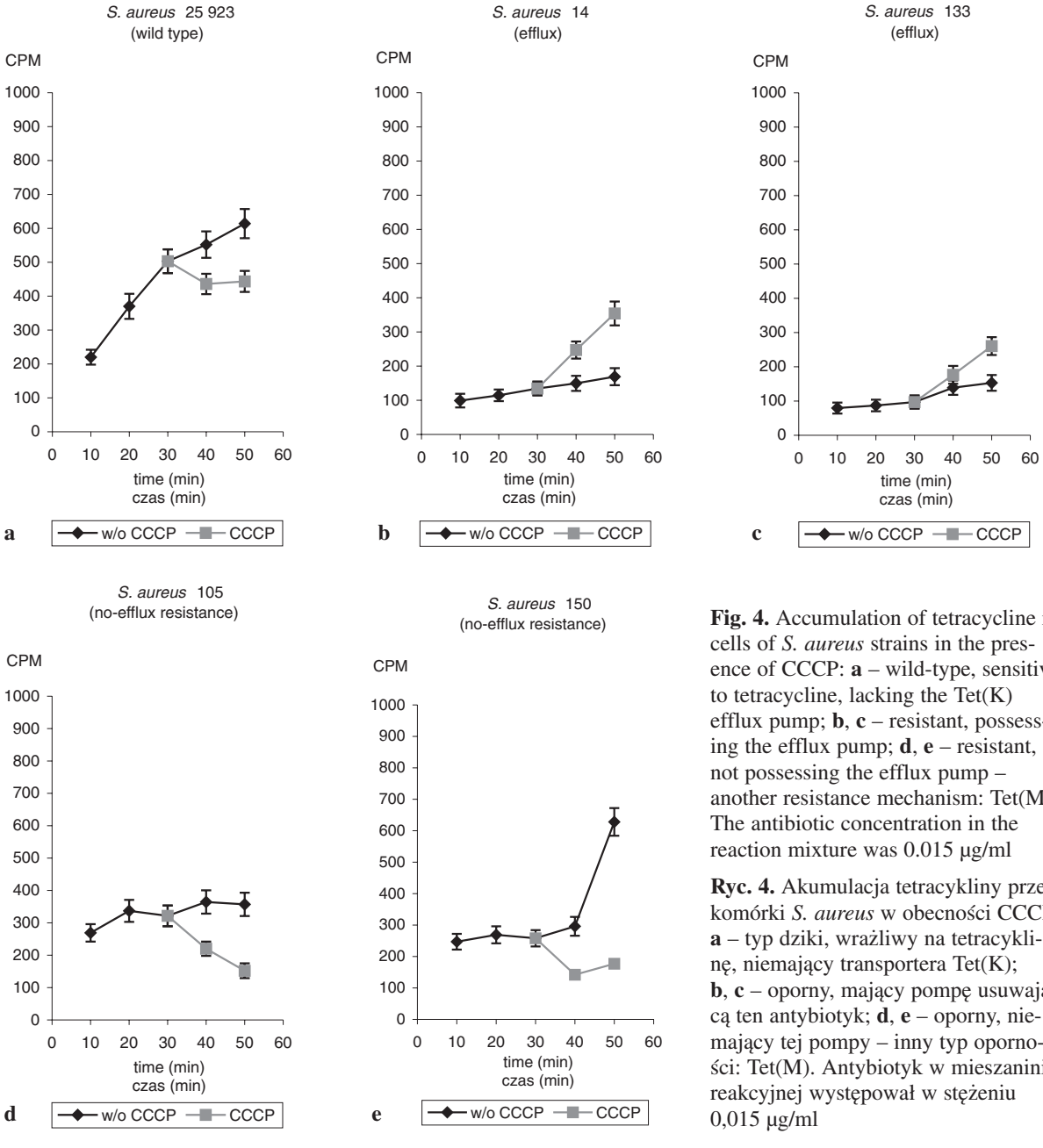

Fig. 4. Accumulation of tetracycline in cells of *S. aureus* strains in the presence of CCCP: **a** – wild-type, sensitive to tetracycline, lacking the Tet(K) efflux pump; **b**, **c** – resistant, possessing the efflux pump; **d**, **e** – resistant, not possessing the efflux pump – another resistance mechanism: Tet(M). The antibiotic concentration in the reaction mixture was 0.015 μg/ml

Ryc. 4. Akumulacja tetracykliny przez komórki *S. aureus* w obecności CCCP: **a** – typ dziki, wrażliwy na tetracyklinę, niemający transportera Tet(K); **b**, **c** – oporny, mający pompę usuwającą ten antybiotyk; **d**, **e** – oporny, niemający tej pompy – inny typ oporności: Tet(M). Antybiotyk w mieszaninie reakcyjnej występował w stężeniu 0,015 μg/ml

In each strain with an active efflux pump, the antibiotic concentration did not change with time or changed insignificantly when CCCP was not present in the reaction mixture. The same was observed in strain SA 105, which was resistant but without the efflux system. In all experiments it was observed that after 10 min of incubation the antibiotic accumulation level in the strains possessing an efflux pump was at least two times lower than in the remaining strains.

Discussion

The most frequent mechanism governing the resistance to tetracycline in the strains under investigation appeared to be the active efflux of the antibiotic performed by the Tet(K) pump (> 90% of strains). This can promote acquiring new resistance determinants (for example *tet(M)*) since the efflux system keeps a certain non-lethal level of antibiotic in the cells, which may favor the development of new resistance mechanisms. Most of the strains possessed only the efflux system as a resistance mechanism. The strains having the Tet(M) system occur infrequently and usually also possess the efflux system. All this would support the supposition that efflux is the original resistance mechanism promoting the selection of various other mechanisms. It is worth noting that presence of both systems (12% of strains) gives the highest level of resistance [1, 2].

The diverse influence of reserpine on the sensitivity of the tested strains to tetracycline probably results from the fact that *S. aureus* strains differ in the amount of genetic efflux determinant in their cells. The more copies of genes coding efflux proteins the cells possess, the higher their observed resistance level [13, 14]. This may explain why one of the strains having the *tet(K)* gene was only intermediate resistant (MIC = 8 μg/ml). In this strain, reserpine lowered the MIC value only by a factor of eight. For much more resistant strains (MIC = 64–128 μg/ml, an expected large number of copies), reserpine decreased MIC 128–256 times.

As for the strains which were still resistant after reserpine treatment, the ribosomal protecting mechanism (TetM) was present in all of them. In almost every strain the efflux system was also present. In these strains, reserpine did not influence the resistance to a significant degree. This may suggest that the ribosomeal protecting mechanism somehow dominates the efflux one.

The molecular study identified only the *tet(K)* and *tet(M)* genes in the strains under investigation. There were no strains found in which *tet(L)* or *tet(O)* were found. These results are supported by other scientific reports in which, apart from *tet(K)* and *tet(M)*, only the *tet(L)* gene was incidentally found in *S. aureus* strains [15].

Table 4 compiles the results on untypical and unexpected types of strains. They indicate that reserpine changed two resistant strains to sensitive ones although a resistance-determining mechanism was lacking. This can be explained by the possible existence of another mechanism causing resistance to tetracycline, one that has not yet been discovered.

The mechanism governing tetracycline removal from *S. aureus* cells depends on the Tet(K) efflux pump, which exchanges the antibiotic for a proton in the antiport mode of translocation [16]. The concentration of antibiotic inside bacterial cells should be relatively low and stable when efflux is active. Since reserpine and CCCP are known to be efflux pump inhibitors, one could expect that in the presence of these factors the tetracycline concentration would increase during incubation inside resistant bacterial cells having the efflux system. CCCP interacts with the cell membrane as an ionophore which dissipates the proton gradient and lowers the proton motive force. This enables protons to move across the plasma membrane without the participation of ATP synthase [17].

The transport of tetracycline into the *S. aureus* cells is dependent on the proton motive force, so CCCP should prevent the postulated active uptake of tetracycline into the cells [9]. The results of the experiments with the two higher concentrations of radiolabeled tetracycline support the hypothesis, showing a decreasing antibiotic accumulation in the strains devoid of the efflux pump. Nevertheless, the results obtained at the lowest extracellular antibiotic concentration throw a new light on the mechanism of tetracycline transport across the membrane. In the wild-type strain *S. aureus* 25923, the resistant strain SA 105 without the efflux pump, and another resistant strain, SA 150, not possessing the efflux system, the antibiotic concentration after treatment with CCCP significantly decreased with time inside the cells. This effect suggests that CCCP not only suppresses the system transporting tetracycline into the cells, but also stimulates a pump that removes this antibiotic in the extracellular direction.

A possible hypothesis that can by formulated at this stage of investigation is that two active tetracycline transport systems may exist in the bacterial cell membrane: one is inhibited by CCCP and the other is stimulated by it. The systems are active to different extents in different strains. In some cases (Table 4), the efflux pump genes are present but

the pump is apparently inactive. A combination of these two systems yields different phenotypes with regard to the sensitivity to tetracycline and the effect of reserpine and CCCP on the accumulation of the antibiotic over time. When only the Tet(K) pump acts, CCCP leads to a rise in the antibiotic's accumulation because the pump is inhibited. In the absence of Tet(K) and the presence of the other pump, stimulated by CCCP, the concentration of the antibiotic decreases, as shown in Figs. 4 a, d, and e. The present authors postulate that the pump stimulated by CCCP is present in the wild-type sensitive strain, but is normally inactive. It is activated by CCCP and reserpine.

Experiments performed with three different concentrations of radiolabeled tetracycline as well as the detection of the amount of antibiotic inside the cells over a 50-min period revealed one more interesting effect: after 40 minutes of cell incubation in the presence of tetracycline at concentrations of 0.044 μg/ml and 0.15 μg/ml, the barrier keeping the antibiotic outside was destroyed, allowing the compound to flow massively into the cells.

The results shown in this paper could be an interesting basis for further investigations in which more detailed characteristics of the tetracycline transport process could be studied on chosen strains with the use of more sophisticated methods.

References

[1] Bryskier A: Tetracyclines. In: Antimicrobial agents – antibacterials and antifungals. Bryskier A (ed.), ASM Press, Washington DC 2005, 1st ed., pp. 642–651.

[2] Chopra I, Roberts M: Tetracycline antibiotics: mode of action, applications, molecular biology and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 2001, 65, 232–260.

[3] Levy SB, McMurry LM, Barbosa TM, Burdett V, Courvalin P, Hillen W, Roberts MC, Rood JI, Taylor DE: Nomenclature for new tetracycline resistance determinants. Antimicrob Agents Chemother 1999, 43, 1523–1524.

[4] Ginn SL, Brown MH, Skurray RA: Membrane topology of the metal-tetracycline/H^+ antiporter TetA(K) from *Staphylococcus aureus*. J Bacteriol 1997, 179, 3786–3789.

[5] Guay GG, Khan SA, Rothstein DM: The *tet(K)* gene of plasmid pT181 of *Staphylococcus aureus* encodes an efflux protein that contains 14 transmembrane helices. Plasmid 1993, 30, 163–166.

[6] Hirata T, Fujihira E, Kimura-Someya T, Yamaguchi A: Membrane topology of the Staphylococcal tetracycline efflux protein Tet(K) determined by antibacterial resistance gene fusion. J Biochem 1998, 124, 1206–1211.

[7] Connell SR, Tracz DM, Nierhaus KH, Taylor DE: Ribosomal protection proteins and their mechanism of tetracycline resistance. Antimicrob Agents Chemother 2003, 47, 3675–3681.

[8] Gibbons S, Udo EE: The effect of reserpine, a modulator of multidrug efflux pumps, on the *in vitro* activity of tetracycline against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) possessing the *tet*(K) determinant. Phytother Res 2000, 14, 139–140.

[9] Hirata T, Wakatabe R, Nielsen J, Satoh T, Nihira S, Yamaguchi A: Screening of an inhibitor of the tetracycline efflux pump in a tetracycline-resistant clinical-isolate of *Staphylococcus aureus* 743. Biol Pharm Bull 1998, 21, 678–681.

[10] National Committee for Clinical Laboratory Standard Institution: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard – Fifth Edition. NCCLS 2000, M7–A5.

[11] Trzciński K, Cooper BS, Hryniewicz W, Dowson HG: Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. J Antimicrob Chemother 2000, 45, 763–770.

[12] Wondrack L, Massa M, Yang BV, Sutcliffe J: Clinical strain of *Staphyloccocus aureus* inactivates and causes efflux of macrolides. Antimicrob Agents Chemother 1996, 40, 992–998.

[13] Reynolds E, Ross JI, Cove JH: Msr(A) and related macrolide/streptogramin resistance determinants: incomplete transporters? Int J Antimicrob Agents 2003, 22, 228–236.

[14] McDevitt D, Wann ER, Foster TJ: Recombination at the coagulase locus in *Staphylococcus aureus*: plasmid integration and amplification. J Gen Microbiol 1993, 139, 695–706.

[15] Schmitz FJ, Krey A, Sadurski R, Verhoef J, Milatovic D, Fluit AC: Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. J Antimicrob Chemother 2001, 47, 239–240.

[16] Jin J, Guffanti AA, Bechhofer DH, Krulwich TA: Tet(L) and tet(K) tetracycline-divalent metal/H^+ antiporters: characterization of multiple catalytic modes and a mutagenesis approach to differences in their efflux substrate and coupling ion preferences. J Bacteriol 2002, 184, 4722–4732.

[17] Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD: 1986. Conversion de L'énergie: mitochondries et chloroplastes. P. 508. In Biologie moléculaire de la cellule. Flammarian-Médecine Sciences, Paris.

Address for correspondence:

Elżbieta Litwinek
Department of Microbiology
Silesian Piasts University of Medicine
T. Chałubińskiego 4
50-368 Wrocław
Poland
Tel.: +48 691 05 25 62, 71 784 13 03
E-mail: litwinekela@wp.pl

Conflict of interest: None declared

Received: 19.09.2007
Revised: 5.10.2007
Accepted: 18.10.2007