

Research article

**THE STRONGEST RESISTANCE OF *Staphylococcus aureus*
TO ERYTHROMYCIN IS CAUSED BY DECREASING UPTAKE
OF THE ANTIBIOTIC INTO THE CELLS**ELŻBIETA PIĄTKOWSKA¹, JERZY PIĄTKOWSKI²
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Abstract: The consequence of excessive use of macrolides is a high occurrence of mechanisms responsible for resistance to these drugs. Of 97 erythromycin-resistant bacterial strains gathered in the Wrocław area in Poland, 60% exhibited very high resistance, and those with the inducible MLS_B (macrolide-lincosamide-streptogramin B) resistance phenotype predominated. Direct genetic investigation revealed that the *erm* genes coding for ribosomal methylases are the most frequently occurring erythromycin resistance-determining genes. No genetic resistance determinant was detected in 13% of the erythromycin-resistant strains. The efflux mechanism occurs in strains isolated from the nasopharyngeal cavity twice as often as in those isolated from other material, where the mechanism connected with target site modification predominates. Measurements of radiolabelled antibiotic accumulation inside bacterial cells revealed that in highly resistant strains (MIC > 1024 µg/ml), an important factor responsible for the resistance is the permeability barrier at the cell wall level. This would be a hitherto unknown mechanism of resistance to erythromycin in *Staphylococcus aureus*.

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Abbreviations used: CCCP – carbonyl cyanide m-chlorophenyl hydrazone; CPM – counts of radioactive decay per minute; MIC – minimum inhibitory concentration; MLS_B CONST. – constitutive resistance to macrolides, lincosamides and streptogramin B; MLS_B IND. – inducible resistance to macrolides, lincosamides and streptogramin B after induction by erythromycin; MS – resistance to macrolides and streptogramin B

Key words: Transmembrane transporters, Antibiotic efflux, Resistance to erythromycin, Drug accumulation, *Staphylococcus aureus*, Ribosomal methylases, *ermA*, *ermC*, *msr(A)*, MLS_B

INTRODUCTION

Staphylococcus aureus is a major pathogen that poses a significant practical and theoretical clinical problem due to its high degree of carriage in the nasopharyngeal cavity, the broad range of infections it causes and, above all, its increasing multidrug resistance [1-3]. Macrolides, which include erythromycin, are one of the antibiotic groups used against infections caused by *S. aureus*. The consequence of their excessive use is the high occurrence of mechanisms responsible for resistance to these drugs. Several mechanisms may be involved in *S. aureus* resistance to macrolides.

The most well-known mechanism, which is also believed to be the most frequently occurring one, is the decreasing affinity of antibiotics for ribosomes due to ribosomal binding site alteration. Ribosomal methylase production, coded by *erm* genes, is involved in this modification [4]. The second described mechanism is the active removal of the antibiotics by efflux systems, which keeps the intracellular antibiotic concentration at a subtoxic level that does not affect bacterial cell growth [5, 6]. In *S. aureus*, such an efflux system is controlled by the Msr(A) pump, which is coded by the *msr(A)* or *msr(B)* gene. The third known cause of resistance in *S. aureus* is the synthesis and activity of macrolide-inactivating enzymes called phosphorylases [7]. In a few strains, gene mutations connected with ribosomal proteins and rRNA have been found. The mutations decrease the susceptibility of these bacteria to this group of antibiotics [8]. A mechanism of resistance caused by a barrier to macrolide permeability has not yet been found in these bacteria, although it is suggested that such a mechanism may play a crucial role in *S. aureus* resistance to acriflavine [9]. In *S. aureus*, the antibiotic efflux mechanism may determine resistance to tetracyclines, macrolides and fluoroquinolones [16, 17]. A study on the frequency of this mechanism in strains isolated in Poland was undertaken because of the lack of published information on the subject. In this paper, we report the results of a study on the mechanisms governing the resistance of *S. aureus* to macrolides. In the study, an as-yet-undescribed cause of resistance in *S. aureus* was identified. This resistance is a consequence of a strong decrease in envelope permeability to erythromycin. This variant of resistance mechanisms turns out to be the most efficient, creating the most resistant strains, with the minimum inhibitory concentration (MIC) value exceeding 1024 µg/ml.

MATERIAL AND METHODS

Ninety-seven strains were isolated from different materials (sputum, urine, upper and lower airway, ear swabs, conjunctival sac swabs, wound swabs, genito-urinary tract swabs) and identified as resistant or intermediate resistant to

erythromycin. The resistance level of *S. aureus* was determined by serial antibiotic dilutions in Mueller Hinton agar and MIC estimation according to the CLSI (Clinical and Laboratory Standards Institute) guidelines [10]. The following criteria were used for erythromycin: MIC \geq 8 $\mu\text{g/ml}$ indicated a resistant strain, MIC = 1-4 $\mu\text{g/ml}$ indicated an intermediate-resistant strain, and MIC \leq 0.5 $\mu\text{g/ml}$ indicated an erythromycin-susceptible strain [11]. The final MIC value was estimated on the basis of at least three measurements. As one of the mechanisms responsible for resistance to this antibiotic may be connected with inducible resistance to lincosamides and streptogramin B, MLS_B (resistance to macrolides, lincosamides and streptogramin B) was also assessed using discs with erythromycin and clindamycine.

In the first stage of the investigation on erythromycin-resistant strains, the known inhibitor of the Msr(A) and Msr(B) pump 2,4-dinitrophenol was used to select those strains in which the pumps are responsible for the resistance. Unfortunately, the highest concentrations used that still enabled survival of *S. aureus* cells turned out to be an inefficient factor in the distinction of pump-determined resistant strains. The MIC value of each strain was comparable, independent of the presence of the pump inhibitor in the growth medium. The identification of the strains with pump-dependent resistance to erythromycin was therefore performed directly by genetic analysis of DNA for the existence of pump-determining genes.

The genetic erythromycin-resistant determinants were detected using polymerase chain reaction (PCR). Amplification was performed according to the formula described by Lina *et al.* [12] with modifications, using the Tag PCR Core Kit (Qiagen). The obtained products were separated in 1.2% agarose gel with ethidium bromide, using a molecular-weight marker. The sizes of the PCR products were 940 bp for *msr(A)*, 595 bp for *msr(B)*, 421 bp for *ermA*, 359 bp for *ermB* and 572 bp for *ermC*.

The erythromycin efflux was measured by monitoring the antibiotic uptake into log-phase *S. aureus* cells according to the formula described by Wondrack *et al.* [13]. The formula was modified in that after 30 min of antibiotic uptake, the reaction mixture was divided into two parts. Into one of the parts, CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) was added at a final concentration of 10 $\mu\text{g/ml}$. The concentration of [¹⁴C]erythromycin was 0.02 $\mu\text{g/ml}$. The radioactivity levels of filtered cells were counted using a Wallac 1409 Liquid Scintillation Counter and shown in CPM (counts of radioactive decay per minute). The sensitive wild strains *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used as a quality control both for the antimicrobial susceptibility testing and the monitoring of antibiotic efflux. Research on erythromycin accumulation was performed in the first instance on resistant strains possessing the genes *msr(A)/(B)*. It was expected that if the pump was active, the antibiotic could be kept at a stable level in the cells, but a rise in accumulation would occur after CCCP inhibitor treatment, which destroys the electrochemical membrane potential [14, 15].

RESULTS

Tables 1 and 2 show that of the 97 strains, 74 are resistant and 23 have decreased sensitivity to erythromycin (MIC = 1-4 µg/ml).

Table 1. Relationship between the erythromycin resistance level and the occurrence of resistance determinants among *S. aureus* strains.

Phenotype of resistance	MIC (µg/ml)	<i>msr(A)/(B)</i> (%)	<i>ermA</i> (%)	<i>ermB</i> (%)	<i>ermC</i> (%)	<i>msr(A)/(B)/ermC</i> (%)	<i>ermA/ermC</i> (%)	Not detected (%)	Number of strains (%)
Resistant	>1024	-	6 (6.2)	1 (1.0)	39 (40.2)	1 (1.0)	3 (3.1)	7 (7.2)	57 (58.8)
	128	3 (3.1)	-	-	-	-	-	-	3 (3.1)
	64	5 (5.1)	-	-	1 (1.0)	-	-	-	6 (6.2)
	32	3 (3.1)	-	-	1 (1.0)	-	-	-	4 (4.1)
	16	2 (2.0)	-	-	1 (1.0)	-	-	-	3 (3.1)
	8	-	-	-	1 (1.0)	-	-	-	1 (1.0)
Intermediate resistant	4	-	7 (7.2)	-	-	-	-	3 (3.1)	10 (10.3)
	2	-	5 (5.1)	-	-	-	-	2 (2.0)	7 (7.2)
	1	-	5 (5.1)	-	-	-	-	1 (1.0)	6 (6.2)
Number of strains (%)		13 (13.4)	23 (23.7)	1 (1)	43 (44.3)	1 (1)	3 (3.1)	13 (13.4)	97 (100)

Table 2. Frequency of erythromycin resistance determinants and range of MIC values in relation to the resistance phenotype of the strains tested.

Phenotype	Range of MIC values	<i>msr(A)/(B)</i> (%)	<i>ermA</i> (%)	<i>ermB</i> (%)	<i>ermC</i> (%)	<i>msr(A)/(B)/ermC</i> (%)	<i>ermA/ermC</i> (%)	Not detected (%)	Number of strains (%)
MLS _B CONST.	32 - 64, >1024	1 (1.0)	5 (5.2)	1 (1.0)	18 (18.6)	-	3 (3.1)	3 (3.1)	31 (32)
MLS _B IND.	1 - 16, >1024	-	18 (18.6)	-	25 (25.8)	1 (1.0)	-	8 (8.2)	52 (53.6)
MS	1 - 2, 16 - 128	12 (12.4)	-	-	-	-	-	2 (2.0)	14 (14.4)

The prevailing populations of resistant strains are those with inducible MLS_B resistance (> 50%), while the smallest group comprises those with the MS phenotype (≈ 14%). The largest group of the resistant strains comprises those with very high resistance, with an MIC of more than 1024 µg/ml (≈ 60%); all of these exhibit the inducible or constitutive MLS_B phenotype. For comparison, MS strains are characterized by a lower resistance level with an MIC in the range of 1-2 and 16-128 µg/ml.

The most frequently occurring genes determining erythromycin resistance are the ones for ribosomal methylase – *erm* (73%). Of these, the *ermC* gene was the

most common (48.4%), followed by *ermA* (26.8%). The *ermB* gene was only identified in one strain. The simultaneous occurrence of two ribosomal methylases in the same strain was equally rare (3.1%). The gene responsible for Msr(A) transporter synthesis was found in 14.4% of the strains under investigation. In 1% of the strains it coexisted with *erm(C)*. No strains were found which had only the *msr(B)* gene, but its presence cannot be excluded in the bacteria possessing the *msr(A)* gene. None of the above-mentioned resistance genes were found in 13% of the strains.

The genes responsible for the active efflux mechanism mainly existed in strains with an MIC value in the range of 16-128 $\mu\text{g/ml}$. The gene *msr(A)* was also found in one strain exhibiting MIC > 1024 $\mu\text{g/ml}$. A similar range of MIC values was noted in the case of strains possessing the *erm(C)* gene (8-64 $\mu\text{g/ml}$, > 1024 $\mu\text{g/ml}$), though the highly resistant strains predominated significantly (> 40%).

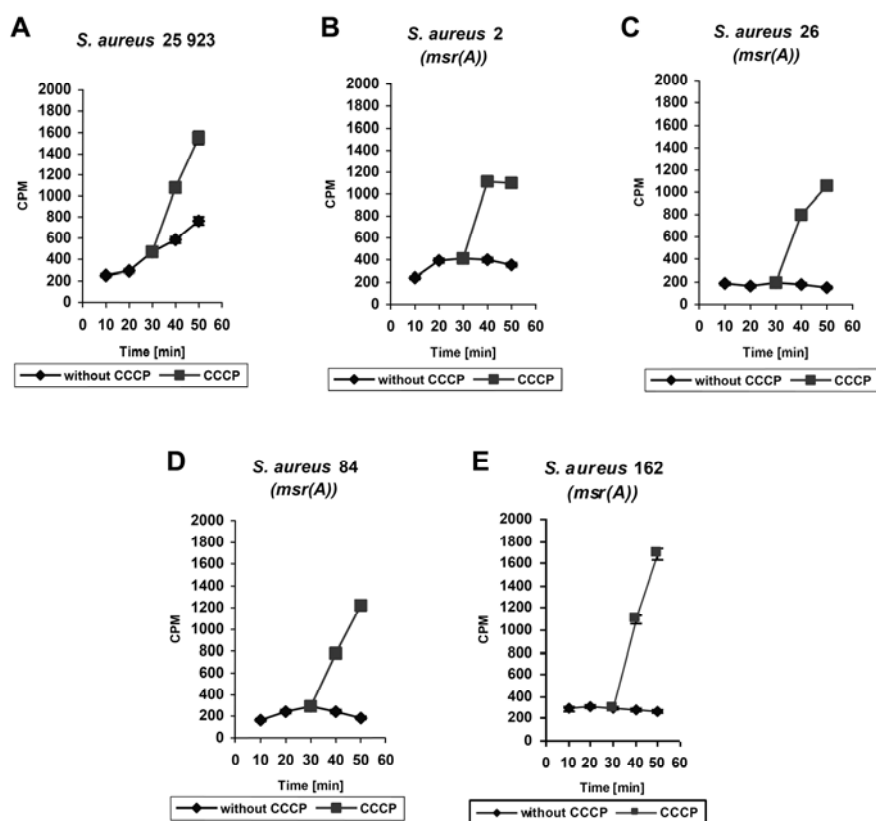


Fig. 1. ^{14}C erythromycin accumulation in *S. aureus* cells with or without the presence of CCCP. A – reference strain, erythromycin susceptible, lacking the *Msr(A)* transporter; B, C, D, E – resistant strains possessing the pump to remove the antibiotic.

The *ermA* determinant was found in strains belonging to two different ranges of MIC: 1-4 $\mu\text{g/ml}$ and $> 1024 \mu\text{g/ml}$. Coexistence of two resistance mechanisms or two different ribosomal methylases conferred a high level of resistance (MIC $> 1024 \mu\text{g/ml}$) to the bacteria. Table 2 shows the dependence of MLS_B resistance on the occurrence of particular genes. In the strains with MS phenotype, *msr(A)* was the only resistance gene found. The *erm* genes predominated in the strains with the phenotypes of constitutive and inducible MLS_B resistance.

The identification of the genetic determinants for the pump involved in the active removal of the antibiotic is not sufficient evidence for efflux as the main mechanism responsible for the resistance. We cannot infer whether the genes are really expressed from their existence alone. In order to obtain direct evidence for their expression, a series of experiments on radioactively labeled antibiotic accumulation was performed to detect the efflux process as a physiological phenomenon.

The results of accumulation tests presented in Fig. 1 A-E support the assumption that the erythromycin efflux system exists in bacterial cells. In the case of all four strains with the *msr(A)* determinant, the antibiotic concentration remained at a similar level throughout the experiment. After CCCP treatment, a drastic increase in the concentration was observed.

At the next stage, similar tests were performed, but on strains in which no *msr(A)* determinant had been identified. These strains exhibited a resistance level in the MIC range 1-2 $\mu\text{g/ml}$. As in the previous experiments, the erythromycin-sensitive *S. aureus* ATCC 25923 strain was used as a control. It was expected that in the case of that group of strains the intensity of accumulation would rise during incubation and it would be strengthened by CCCP addition.

The results obtained in these experiments supported our expectations. As we can see in Fig. 2 A-E, there was a mild growth of the accumulation curve for the variant without the inhibitor, and significantly more intensive growth after CCCP was added to the reaction mixture. The most interesting results were provided by the study on highly resistant *S. aureus* strains with an MIC $> 1024 \mu\text{g/ml}$, in which, with one exception, no efflux determinants were found. The ribosomal methylase gene *ermC* was found most frequently in these strains, although there were also strains in which, in spite of such a high resistance level, no genetic determinants for erythromycin resistance were found. The results are presented in Fig. 3 A-E.

These results show that in all of the strains, the antibiotic concentration level, expressed as the counts of radioactive decay per minute, remained at a very low level (50–100 CPM) during 50 min of cell incubation in an isotopic environment. Furthermore, the antibiotic accumulation was not significantly modified by CCCP, as observed for strains exhibiting a low resistance level.

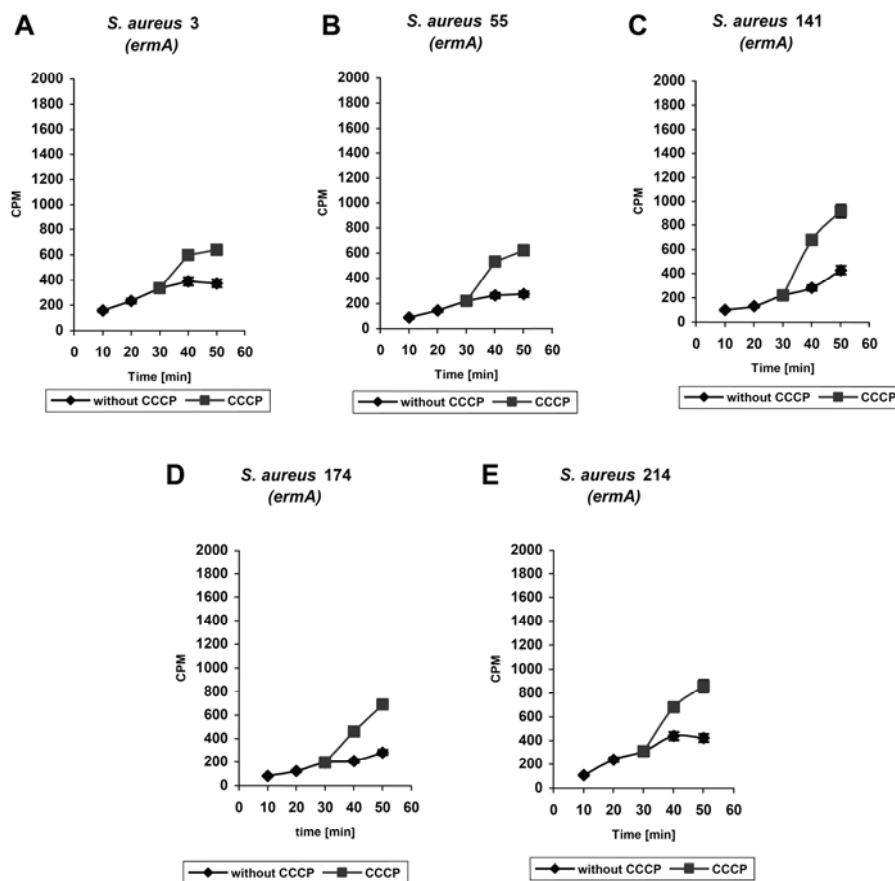


Fig. 2. $^{[14]}$ erythromycin accumulation in *S. aureus* cells with or without CCCP. A, B, C, D, E – resistant strains possessing the ribosomal methylase gene (*ermA*) but devoid of the Msr(A) transporter.

The persistence of such a low antibiotic concentration during the period of the experiment may indicate the activity of a new, as-yet-undescribed resistance mechanism connected with antibiotic uptake limitation, probably in the cell wall. The fact that the inhibitor did not significantly change the intensity of the accumulation seems to support the suggestion.

If we assume that a barrier in the cell wall may be involved in the erythromycin resistance level in strains with an MIC > 1024 $\mu\text{g/ml}$, it would be interesting to verify whether this mechanism may be involved in the resistance of other strains with a lower MIC value. If the resistance of these strains depends on the intensity of antibiotic uptake, there should be a correlation between the MIC value and the slope for the erythromycin accumulation curve. In order to show

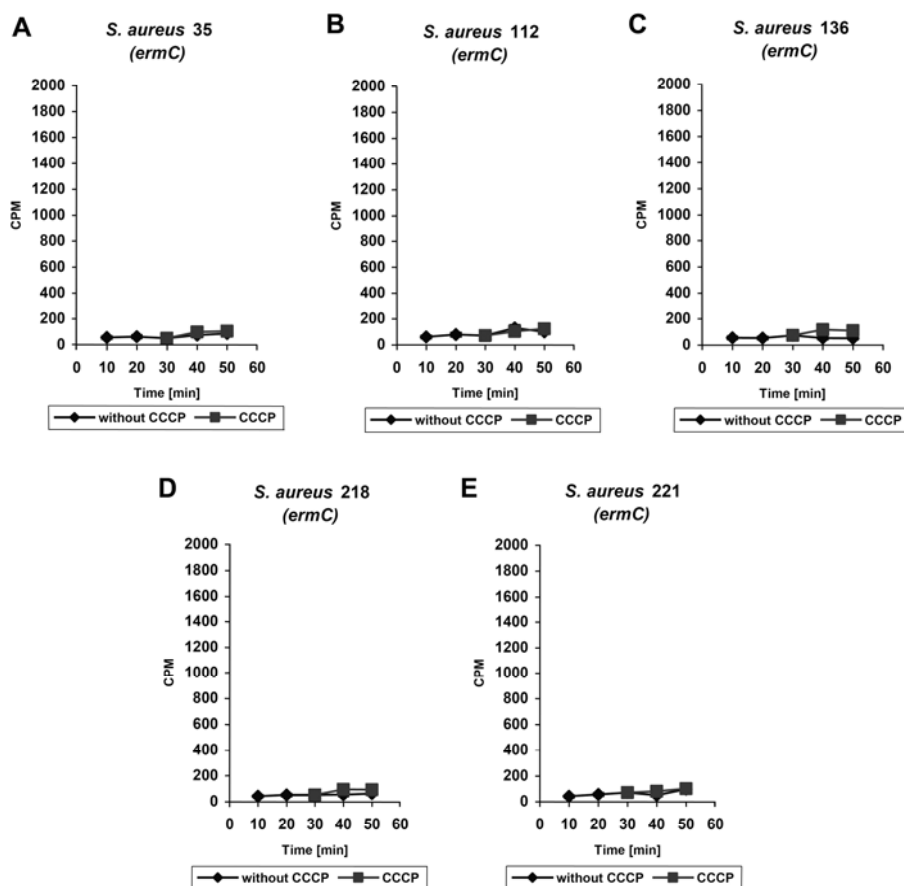


Fig. 3. ^{14}C erythromycin accumulation in *S. aureus* cells with or without CCCP. A, B, C, D, E – highly-resistant strains lacking the Msr(A) transporter but possessing the ribosomal methylase gene (*ermC*).

this, these slopes were established for strains with different MIC values. We expected that the lower the slope value is, the higher the MIC value should be. The results presented in Fig. 4 are persuasive that such a correlation really occurs. The mean slope value decreased with increasing MIC value.

The presented slope values concern erythromycin accumulation without the inhibitor. As we know, the uptake of the antibiotic is more intense with the CCCP inhibitor, so we assumed that the method for the accumulation rate would be more sensitive in the presence of this inhibitor. Therefore, we decided to test the same strains with CCCP but, for practical reasons, with a shorter accumulation time (14 min.).

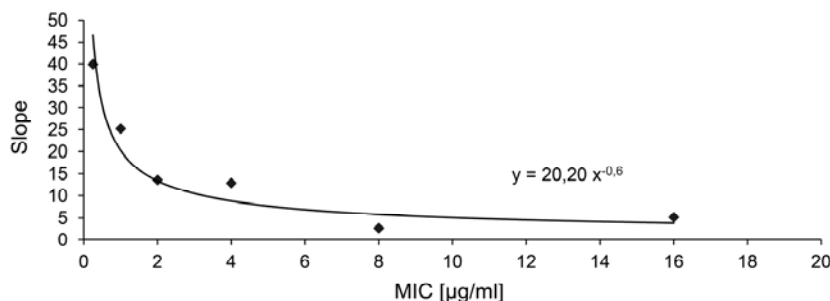


Fig. 4. Relationship between the MIC value for erythromycin and the slope value for erythromycin accumulation in *S. aureus*.

The results presented in Fig. 5 again support the correlation between the MIC value and the accumulation rate. Fig. 4 shows that the slope value is negatively correlated with the MIC value. For low MIC values the accumulation rate changes significantly, but at higher MIC values the changes of the slope are less significant.

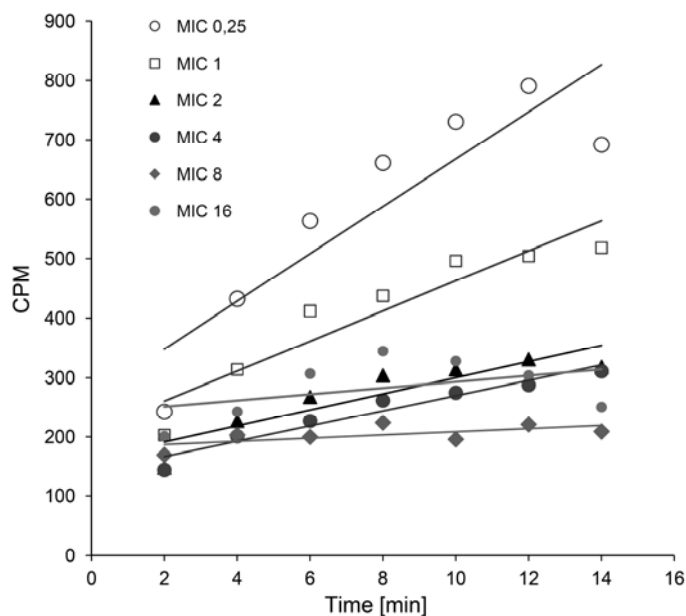


Fig. 5. Slopes for ^{14}C erythromycin accumulation curves for *S. aureus* strains in the presence of CCCP.

DISCUSSION

Ninety-seven erythromycin-resistant *Staphylococcus aureus* strains gathered in the Wrocław area were tested for their resistance mechanisms. It was not the aim of our research to detect the proportion of resistant strains in all isolates but only

the proportion of strains having an active efflux system for erythromycin. Therefore, we only dealt with strains that had been previously identified as resistant to the antibiotic. The resistance level varied in the range from MIC = 1 µg/ml to MIC > 1024 µg/ml. Each resistant variant with an MIC value lower than 1024 µg/ml was represented by just a few strains, whereas nearly 60% of the strains exhibited very high resistance. Furthermore, strains with the inducible MLS_B resistance phenotype predominated. This result differs to that obtained by Lina *et al.* [12], who found that more strains possessed the constitutive phenotype of erythromycin resistance in research undertaken in an area of France.

The main purpose of our study was to identify the activity of the efflux mechanism in our strains. The 2,4-dinitrophenol compound, postulated as a membrane Msr(A) and Msr(B) pump inhibitor [18], turned out to be inefficient in differentiating the strains possessing or lacking the efflux system. The strains were as resistant in the medium containing the inhibitor as they were in the medium without it, making it impossible to select strains suspected of having the efflux pump on the basis of MIC values using 2,4-dinitrophenol as an inhibitor. Direct genetic investigations reveal that the *erm* genes coding for ribosomal methylases are the most frequently occurring erythromycin resistance-determining genes. Similar data were obtained in other studies [12, 19, 20]. Different papers report on *erm(A)* or *erm(C)* domination. We found a general predominance of the *erm(C)* gene. However, this gene mostly occurred in highly resistant strains, whereas the *erm(A)* gene predominated in less resistant ones. The *erm(B)* gene was identified in just one strain. The frequency of this determinant in *S. aureus* and coagulase-negative staphylococci is low, which is supported by Lina *et al.* [12]. Coexistence of two different ribosomal methylases was recorded in only three strains, which correlates with data in the literature [20]. The *Msr(A)* gene was found in 14% of strains, as compared with 2.1% of such strains identified by Lina *et al.* [12].

It seems to be worth noting that no genetic resistance determinant was detected in 13% of erythromycin-resistant strains. Previous investigations revealed that rare macrolide resistance mechanisms in *S. aureus* are connected with a mutation in the 23S rRNA subunit and with enzymatic inactivation of the antibiotic by phosphotransferase coded by the *mph(C)* gene [8, 13, 21, 22]. Research to identify those mechanisms was not carried out in our study but if those 13% of strains represented the type of resistance connected with enzymatic inactivation, it would be more frequent than has been thought so far [8]. Thus, we postulate that in those 13% of strains, a hitherto unknown mechanism is responsible for erythromycin resistance in *S. aureus*.

More direct investigations on membrane transporter activity were carried out at the physiological level, using measurements of radiolabelled antibiotic accumulations inside bacterial cells. In the presence of CCCP, a rapid increase in the erythromycin concentration appeared, both in resistant strains and in the reference one. This can be explained by an increased permeability in the plasma membrane, caused by this inhibitor as a consequence of membrane potential

neutralization [14, 15]. This potential creates a barrier for charged compounds such as erythromycin. Since the drastic increase in erythromycin concentration with CCCP has been observed both in control strains and in those possessing these pumps, it is not a significant effect for efflux pump detection.

In strains possessing an efflux pump, antibiotic accumulation was limited significantly in the absence of CCCP. The increase in concentration over time was more intense in the control strain and in every strain with a different, non-“efflux” resistance mechanism. However, the slopes of the accumulation curves for the non-“efflux”-resistant strains were lower than those of the control one. One should expect that the slopes would be of similar shape if the efflux were the only mechanism participating in the antibiotic accumulation. Therefore, lower slopes in resistant strains might indicate that another mechanism is involved in the accumulation process.

The tests with the highly resistant strains (MIC > 1024 µg/ml) seem to support this strongly: the accumulation measurements reveal that an important factor limiting erythromycin uptake by *S. aureus* cells is the permeability barrier at the cell wall level. This is the most obvious inference from the accumulation being almost completely stopped in those strains, even when CCCP was added to the reaction mixture. In our opinion, the observations of the accumulation level in the highly resistant strains are the most convincing evidence for that, but we decided to also consider less direct but suggestive observations. Among other observed behavior, the cells of highly resistant strains tended to form larger and more stable aggregates, which indicates that they differ in cell wall composition from the less resistant ones.

It was difficult to accept that such strong resistance could be caused by plasma membrane modification. This would require such considerable changes that the function of the membrane would be destroyed. We postulate the alteration of the cell wall rather than of the plasma membrane as a reason for the high resistance phenomenon.

If the resistance is genetically dependent, one can expect that a rapid increase in resistance would appear in strains possessing this gene as compared with those not possessing it. Such an increase is observed among strongly resistant and less resistant strains. On the other hand, if the resistance is polygenic, a linear distribution of the resistance level could be expected. This seems to be observed in less resistant strains. The negative correlation between the MIC values and accumulation slopes may reflect changes in antibiotic permeability as one of the causes of resistance to erythromycin.

The highly resistant strains (MIC ≥ 1024 µg/ml) may contain different *erm* genes, *ermC* being more frequent. However, both *erm* and *msr* genes were absent in some highly resistant strains. This indicates that these genes are probably not involved in the mechanism that gives such high resistance.

The limitation of antibiotic uptake to *S. aureus* cells, which according to our hypothesis is the main reason for resistance in the highly resistant strains, is a very important factor exactly because it concerns the more resistant strains.

It is possible that the barrier in permeability may also cooperate with other mechanisms, giving the linear shape of the relationship between the MIC and accumulation slope values in less resistant strains. Further research on the most highly resistant strains is needed, especially experiments concerning cell wall composition and the genetic determinants responsible for such strong resistance. Successful selections and detections for membrane molecular targets are possible [23, 24].

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