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Characteristics of *Staphylococcus aureus* Strains Isolated in Poland in 1996 to 2004 That Were Deficient in Species-Specific Proteins[▽]

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One hundred seventy *Staphylococcus aureus* isolates, collected in 1996 to 2004, were reidentified by phenotypic and genotypic methods. One hundred ten of these (65%) were confirmed, as previously denoted, to be clumping factor (CF)- or free coagulase-deficient *S. aureus*, based on their phenotype. Based on the CF or coagulase production, three groups of phenotypically deficient *S. aureus* isolates were distinguished. Group 1 encompassed CF-positive and coagulase-deficient isolates, group 2 consisted of CF-deficient and coagulase-positive isolates, and group 3 included isolates that were CF positive, had delayed coagulase activity, and were deficient in other species-specific features. All investigated strains harbored the *clfA*, *clfB*, *coa*, *spa*, and *nuc* genes, but the presence of their products was not detected by the phenotypic methods. Glycopeptide susceptibility testing showed that 26 isolates (23.6%) were hetero-glycopeptide-intermediate *S. aureus* (hGISA) or hetero-teicoplanin-intermediate *S. aureus* (hTISA), based on the population analysis profile. The relatedness of the isolates was evaluated by multiple-locus variable number of tandem repeats analysis, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing. The phenotypically deficient *S. aureus* isolates were classified into PFGE types B (ST239-III) and D (ST246-IA) and were related to the common clones, Hungarian and Iberian, respectively, which have been widely disseminated in Poland and globally. The simultaneous occurrence of hGISA/hTISA and the CF-deficient phenotypes was found for 62.1% of isolates belonging to group 2. The majority of these isolates were assigned to the Iberian clone (PFGE type D; ST247-IA). An association between the defect in coagulase and that in thermonuclease production was observed, which concerned 59.2% of isolates of group 1. The majority of these isolates belonged to the Hungarian clone (PFGE type B; ST239-III).

Staphylococcus aureus is one of the most important human pathogens associated with hospital and community-acquired infections. Due to its clinical importance, misidentification of this pathogen in clinical samples can have serious consequences in increased morbidity and mortality. *S. aureus* produces a large number of virulence factors, which include surface proteins involved in colonization and immune evasion, as well as extracellular toxins responsible for tissue destruction and inactivation of host defense mechanisms (4, 33). Classic identification of *S. aureus* is based on its ability to clump in plasma via the activity of clumping factor (bound coagulase) (CF) and coagulase (free coagulase) (4, 48). The simultaneous production of CF and coagulase is a characteristic feature of most *S. aureus* isolates and plays an important role in species classification and microbial diagnosis (4, 29). Other proteins whose activities are used for phenotypic identification of staphylococcal species are protein A and the less species-specific thermonuclease (4).

The two CF protein variants, ClfA and ClfB, show similar molecular organization and high sequence homology and are encoded by the *clfA* and *clfB* genes, respectively (27, 30). ClfA is responsible for nonenzymatic *S. aureus* clumping in plasma and was shown to promote adherence of bacterial cells to fibrinogen-coated surfaces in vitro and in experimental animal models in vivo (26). The *coa* gene codes for another protein used in phenotypic identification of staphylococci, coagulase. The staphylocoagulase tube test is still the "gold standard" in clinical diagnostics of *S. aureus*, though certain other staphylococcal species that are also coagulase positive are rarely identified in human infections (4, 53). Coagulase is produced extracellularly by *S. aureus* cells and reacts with prothrombin, simultaneously transforming it into staphylothrombin. This complex acts directly on fibrinogen, converting it to fibrin, which results in plasma clotting. Coagulase can also react with fibrinogen independently on prothrombin (27). There may be different reasons for a phenotypic lack of activity of species-specific *S. aureus* characteristics, including, e.g., DNA mutations, DNA rearrangements due to insertions or deletions, or the influence of different regulatory systems on the expression of particular molecular factors or pathways (31, 48, 50). The aim of this study was to characterize, both phenotypically and genetically, clinical isolates of *S. aureus* deficient in CF or coagulase production.

MATERIALS AND METHODS

Bacterial isolates. In total, 170 (7%) clinical isolates of *S. aureus* were selected from 2,443 isolates collected by the National Institute of Public Health in Warsaw in several surveys between 1996 and 2004. The 170 isolates came from 41 different medical centers in Poland, as well as from single centers in three other countries (Slovenia, Hungary, and Bulgaria). The study isolates were initially defined as phenotypically problematic and were sent to the National Institute of Public Health for species confirmation. Most of the isolates were recovered from

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blood, skin, and soft-tissue infections and from other clinical sources, including nasal swabs, urine, and bronchoalveolar lavage. The origins of 19 isolates were unknown.

Scheme of *S. aureus* species identification. *S. aureus* species identification was based on phenotypic criteria including colony morphology and the presence all of the characteristic features in the following conventional tests: CF, coagulase in rabbit plasma, thermonuclease, and mannitol fermentation (4, 29). In cases with a lack of CF or coagulase activity, we called the *S. aureus* isolates phenotypically deficient. These isolates were further identified to the species level by using *clfA*, *clfB*, *coa*, *nuc*, and *spa* gene determination (6, 19, 37). Finally, the presence all of these molecular markers determined the *S. aureus* species affiliations of isolates tested in this study.

Phenotypic methods. (i) Antimicrobial susceptibility testing. Susceptibilities to the following antimicrobial agents were determined by the disk-diffusion method: cefoxitin, penicillin, clindamycin, erythromycin, lincomycin, streptomycin, gentamicin, kanamycin, tobramycin, tetracycline, doxycycline, trimethoprim-sulfamethoxazole, spiramycin, ciprofloxacin, fusidic acid, chloramphenicol, rifampin, and mupirocin (200-μg disc). For the majority of antimicrobials, the results were interpreted according to the CLSI criteria (8), except in the cases of fusidic acid, lincomycin, and streptomycin, for which the French guidelines (44) were adopted. Mupirocin susceptibility was evaluated as described by Finlay et al. (14). *Staphylococcus aureus* ATCC 29253 was used as the quality control strain. The methicillin-resistant *S. aureus* (MRSA) phenotype was confirmed by *mecA*-specific PCR (32).

(ii) Glycopeptide susceptibility testing. The strategy for glycopeptide susceptibility testing in this study was performed as described previously (22), including screening according to the methods of Trakulsomboon et al. (47), using brain heart infusion agar supplemented with 4 μg/ml or 6 μg/ml vancomycin according to CLSI methods (8). MICs of vancomycin and teicoplanin were evaluated according to CLSI guidelines (8). Etest macromethod values were determined as described previously (52) for heterogeneous glycopeptide resistance detection by using a heavy inoculum (2 McFarland units). A population analysis profile (PAP) done according to the method of Trakulsomboon et al. (47) was used to confirm the hetero-glycopeptide-intermediate *S. aureus* (hGISA)/hetero-teicoplanin-intermediate *S. aureus* (hTISA phenotype for isolates with the presence of a subpopulation of cells (around 10^{-6}) which were grown at a vancomycin concentration of 4 μg/ml (18) or more and a teicoplanin concentration of 8 μg/ml or more. PAP analysis was performed for all isolates suspected of having the hGISA/hTISA phenotypes based on the screening and Etest macromethod results described above. *Staphylococcus aureus* ATCC 29213, susceptible to vancomycin (GSSA); *S. aureus* ATCC 700698 (Mu3), hGISA; and *S. aureus* ATCC 700699 (Mu50), glycopeptide-intermediate *S. aureus* (GISA), were used as reference strains.

(iii) Delta-hemolysin expression. The functionality of the *agr* operon was assessed by observation of delta-hemolysin production as described previously (40). This was determined using *S. aureus* ATCC 25923, a strain that produces a large zone of beta-hemolysis without the interference of alpha- or delta-hemolysins (15). Delta- and beta-hemolysins act synergistically in the lysis of sheep red blood cells. Delta-hemolysin produced by a test strain results in an enhanced zone of hemolysis in areas where this lysis overlaps with the beta-hemolysis zone of ATCC 25923.

Molecular methods. (i) Preparation of total DNA for PCR. Total DNA of the isolates was purified using the Genomic Mini DNA kit (A&A Biotechnology, Gdynia, Poland) as previously described (37).

(ii) MLVA. Preliminary typing was performed by the PCR-based multiple-locus variable number of tandem repeats analysis (MLVA) method previously described by Sabat et al. (37). MLVA types were denoted by capital letters, with subtypes indicating closely related strains assigned by additional numbers. Clonality analysis of the isolates was performed according to criteria previously described (25).

(iii) PFGE. Total DNA preparation, digestion with the SmaI restriction enzyme (MBI Fermentas, Vilnius, Lithuania), and pulsed-field gel electrophoresis (PFGE) (42) were performed as described by Chung et al. (7). A CHEF DR III system (Bio-Rad, Hercules, Calif.) was used for electrophoresis. Restriction patterns were compared using Molecular Analyst Software Fingerprinting, version 1.12 (Bio-Rad), and were classified into PFGE types and subtypes according to the criteria of Tenover et al. (46).

(iv) MLST. Multilocus sequence typing (MLST) analysis was performed as described by Enright et al. (12). Allele sequences of the seven loci were compared to those submitted to the *S. aureus* MLST database (www.mlst.net). The sequence type (ST) of each isolate was defined by an allelic profile that was composed of seven allele numbers.

(v) SCC*mec* analysis. The staphylococcal cassette chromosome *mec* (SCC*mec*) types were determined by multiplex PCR as described by Oliveira and de Lencastre (32). The following reference strains were used in the analysis: the Iberian clone PER 184 and PER 88 isolates (32) for SCC*mec* type I and its IA variant, respectively; the New York/Japan strain Mu 50 (3) for SCC*mec* type II; the Hungarian strain J405 (www.mlst.net) and the Brazilian strain HU25 (32) for SCC*mec* type III and its IIIA variant, respectively; and the pediatric strain POL3 (32) for SCC*mec* type IV.

(vi) *agr* typing. The accessory gene regulator (*agr*) group (alleles) was determined by the multiplex PCR strategy as described by Lina et al. (24).

RESULTS

Phenotypic methods. (i) Species identification. After reidentification with standardized phenotypic methods, 110 out of the 170 isolates (65%) were confirmed as phenotypically deficient *S. aureus*. The remaining isolates were classified as *S. aureus* with normal phenotypic characteristics or as different staphylococcal species. Among phenotypically deficient *S. aureus* isolates, three different groups were distinguished, based on the phenotypic tests described above; group 1 ($n = 54$) encompassed CF-positive [CF(+)] and free coagulase-deficient [COA(−)] isolates (Table 1); group 2 ($n = 37$) included CF-deficient [CF(−)] and coagulase-positive [COA(+)] isolates (Table 1);and group 3 ($n = 19$) isolates consisted of isolates that were CF(+), showed delayed coagulase activity, and were phenotypically defective in other *S. aureus* species-specific features, such as thermonuclease production [NUC(−)] and mannitol fermentation [MAN(−)] (Table 1).

(ii) Susceptibility testing. Among all of the phenotypically deficient isolates, 77 (70%) were characterized as MRSA. However, the frequencies of MRSA isolates varied among the three distinguished groups, accounting for 75.9%, 83.7%, and 26.3% of the isolates, respectively. MRSA isolates from all three groups demonstrated multidrug resistance comprising resistance to all aminoglycosides as well as to erythromycin, spiramycin, lincosamides, ciprofloxacin, tetracycline, and doxycycline.

(iii) Susceptibility to glycopeptides. Twenty-seven isolates (24.5%) were identified as hGISA/hTISA by the Etest macromethod, confirmed by PAP (Table 1). The majority of these ($n = 24$) were assigned to phenotype group 2 [CF(−), COA(+)]. MICs of 0.5 to 2 μg/ml of vancomycin and 1 to 16 μg/ml of teicoplanin were determined by the CLSI microdilution method. All isolates were classified as susceptible or intermediate to glycopeptides according to the CLSI criteria (8). Using the Etest macromethod with vancomycin and teicoplanin, inhibitory concentrations of 4 to 24 μg/ml and 12 to 24 μg/ml, respectively, were obtained. The PAP study revealed that cultures of these isolates contained fractions of cells that grew at vancomycin concentrations of 4 to 8 μg/ml and teicoplanin concentrations of 16 to 32 μg/ml, with a frequency of approximately 10^{-6}. According to the criteria of Trakulsomboon et al. (47), these isolates were classified as hGISA/hTISA. Isolates that grew at teicoplanin concentrations of 16 to 32 μg/ml but without reduced susceptibility to vancomycin were identified as hTISA.

(iv) Phenotype coincidence. The simultaneous occurrence of the hGISA/hTISA and CF-deficient phenotypes was observed for 62.1% ($n = 23$) of isolates belonging to phenotype group 2 (Table 1). Moreover, an association between the defect in coagulase and that in thermonuclease production was observed

TABLE 1. A. Genotypic and phenotypic characteristics of clumping factor- or coagulase-deficient *S. aureus* strains

Strain group[a]	PFGE type (n^b)	PFGE subtype (n^b)	Genotype					Phenotype (n^b)			Clone characteristics		
			ST	SCC*mec* type	Presence of *clfA*, *clfB*, *coa*, *spa*, and *nuc* genes (n^b)	Clonal complex	*agr* group	Methicillin resistance phenotype	Glycopeptide resistance phenotype[c]	Delta-hemolysin production[d]	Relatedness to Polish and international *S. aureus* clones (reference[s])[e]	Center code[f]	Yr or period of isolation
1	A (1)	A1 (1)	45	IV	+ (4)	CC45	I	MRSA	GSSA	+	HeMRSA-Pol4 (23), Berlin clone (36)	35	2001
	A (3)	A4 (2), A13 (1)	45			CC45	I	MSSA	GSSA	+		7, 19	1999–2004
	B (34)	B5 (16), B8, B46, B47, B48 (3), B49, B50 (4), B51, B52, B53, B54, B55, B56, B57	239, 241	III, IIIB, IIIC	+ (34)	CC8	I	MRSA	GSSA; hGISA (2)	+ (32); − (2)	HoMRSA-Pol1 (23), Hungarian clone (9)	1, 3, 6, 14, 19, 20, 22, 25, 26, 27, 31 40, 41	1997–2003
	C (2)	C4	239	III	+ (2)	CC8	I	MRSA	GSSA	+	He/HoMRSA-Pol1 (23), Brazilian clone (2)	1	1997
	K (2)	K17, K18 (2)	239, 254		+ (5)	CC8	I	MSSA	GSSA	+		26, 41	1998–2001
	K (3)	K19, K21	239	IIIC	+ (3)	CC8	I	MRSA	GSSA	+	HoMRSA-Pol3 (23)	5, 25	1999, 2003
	R (3)	R1	15		+ (3)	CC15	II	MSSA	GSSA	+	Polish MSSA clone	15, 23, 37	1996, 1999, 2002
	N (1)	N9	30		+	CC30	III	MSSA	GSSA	+	HeMRSA-Pol3 (23), SWP[4] clone (36, 49)	10	1997
	F (1)	F14	5	IV	+	CC5	II	MRSA	GSSA	+	HeMRSA-Pol1 (23), pediatric MRSA clone (23, 33)	35	2001
	G (1)	G1	80	IV	+	Singleton	III	MRSA	GSSA	+	HeMRSA-Pol2 (23), Mediterranean clone (1, 17)	7	1996
	RR (1)	RR1	121		+	CC51	IV	MSSA	GSSA	+		16	1999
	Y (1)	Y2	395		+	Singleton	I	MSSA	GSSA	+	Sporadic	35	1999
	ZZ (1)	ZZ1 (1)	7		+	Singleton	I	MSSA	GSSA	+	Sporadic	4	2002
2	D (30)	D1, D6 (3), D7 (23), D27, D30 D32	247	IA	+ (30)	CC8	I	MRSA	hGISA (23); GSSA	− (22), + (8)	HoMRSA-Pol2 (23), Iberian clone (33)	4, 5, 8, 9,10, 11, 12, 13, 15, 19, 21, 24, 28, 29, 30, 31, 32, 33, 34, 35	1996–2004
	N (1)	N9 (1)	30		+	CC30	III	MSSA	GSSA	+	HeMRSA-Pol3 (23), SWP[4] clone (36, 49)	36	1998
	R (3)	R1, R2, R3	15		+ (3)	CC15	II	MSSA	GSSA	+	Polish MSSA clone	38, 35	2002, 2000 1996
	K (1)	K23	239	III	+	CC8	I	MRSA	hTISA	−	HoMRSA-Pol3 (23)	2	1998
	WW(1)	XX1	707		+	Singleton	III	MSSA	GSSA	+	Sporadic	23	1996
	YY (1)	YY1	182		+	Singleton	I	MSSA	GSSA	+	Sporadic	10	1997
3	A (1)	A1 (1)	45	IA	+	CC45	I	MRSA	GSSA	+	HoMRSA-Pol4 (23), Berlin clone (36)	38	
	D (3)	D30, D7 (2)	247	IA	+	CC8	I	MRSA	hTISA (1), GSSA (1)	− (1), + (1)	HoMRSA-Pol2 (23), Iberian clone (33)	5, 31	
	R (3)	R1	15		+ (3)	CC15	II	MSSA	GSSA	+	Polish MSSA clone	13, 7, 18	
	N (3)	N8, N10, N11	30		+	CC30	III	MSSA	GSSA	+	HeMRSA-Pol3 (23), SWP clone (36, 49)	16, 38	

XX (3)	XX1	1		+	CC1	II	MSSA	GSSA	+		7, 35, 27
T (1)	T1	1		+	CC1	III	MSSA	GSSA	+		35
K (1)	K22	239		+	CC8	I	MSSA	GSSA	+		17
RR (1)	RR2	121		+	CC51	IV	MSSA	GSSA	+	Sporadic	35
NY (1)	NY1	5	I	+	CC5	II	MRSA	GSSA	+	EMRSA-3 (36)	16
Y (1)	Y1	395		+	Singleton	II	MSSA	GSSA	+	Sporadic	12
X (1)	X2	446		+	Singleton	III	MSSA	GSSA	+	Sporadic	7

[a] Group 1, based on phenotypical tests: CF(+), COA(−), n = 54; NUC(−), n = 32; NUC(+), n = 22; MAN(−), n = 1; MAN(+), n = 53. Group 2, based on phenotypical tests: CF(−), COA(+), n = 37; NUC(−) n = 1; NUC(+), n = 36; MAN(−), n = 1; MAN(+), n = 36. Group 3, based on phenotypical tests: CF(+), delayed coagulase, COA(+), n = 19; NUC(−), n = 10; NUC(+), n = 10; MAN(−), n = 0; MAN(+), n = 19.

[b] *n*, number of isolates.

[c] The PAP method was used to confirm the hGISA/hTISA phenotype.

[d] +, enhanced zone of hemolysis created by the interaction of beta-hemolysin of ATCC 25923 and delta-hemolysin of the test strain; −, lack of enhanced zone of hemolysis (lack of delta-hemolysin production).

[e] HeMRSA and HoMRSA are Polish clones. SWP, Southwest Pacific.

[f] Center code number reflects the locations of Polish cities or foreign countries of origin of isolates, listed here in alphabetical order: 1, Bielsko Biala; 2, Bulgarian; 3, Bydgoszcz; 4, Bytom; 5, Czestochowa; 6, Gdansk; 7, Grajewo; 8, Hungary; 9, Kalisz; 10, Konin; 11, Koszalin; 12, Krakow1; 13, Krakow2; 14, Lomza; 15, Makow Maz.; 16, Mlawa; 17, Nowa Sol; 18, Olsztyn; 19, Otwock; 20, Poznan; 21, Rzeszow; 22, Siemianowice Sl.; 23, Sieradz; 24, Slovenia; 25, Sochaczew; 26, Suwalki; 27, Szczecin; 28, Warsaw, Waw1; 29, Waw2; 30, Waw3; 31, Waw4; 32, Waw5; 33, Waw6; 34, Waw7; 35, Waw8; 36, Waw9; 37, Wejherowo; 38, Wolomin; 39, Zabrze; 40, Zamosc; 41, Zielona Gora.

for 59.2% ($n = 32$) of isolates belonging to phenotype group 1 (Table 1).

(v) Delta-hemolysin expression. Only for isolates with the hGISA/hTISA phenotype was the production of delta-hemolysin not observed (Table 1).

Molecular methods. (i) Molecular identification. The presence of the *clfA*, *clfB*, *coa*, *spa*, and *nuc* genes was determined for all of the 110 phenotypically deficient isolates by PCR (Table 1).

(ii) PFGE and MLVA typing. One hundred ten phenotypically deficient *S. aureus* isolates were grouped into 18 PFGE types (Table 1), with the most common types, B ($n = 34$; 14 subtypes) and D ($n = 33$; 6 subtypes), predominating among isolates belonging to phenotype groups 1 and 2, respectively. Phenotype group 3 was much more clonally diverse, with 11 PFGE types distinguished. In general, PFGE segregated MRSA from methicillin-susceptible *S. aureus* (MSSA) isolates except for PFGE types A and K, which grouped as both MRSA and MSSA isolates. The genetic relatedness between MRSA and MSSA was previously determined for the Polish *S. aureus* isolates (J. Krzyszton-Russjan, personal communication), as well as for those from the other countries (36). Only three PFGE types, D ($n = 24$), B ($n = 2$), and K ($n = 1$), were discerned among the hGISA/hTISA isolates. The majority of these ($n = 24$) were assigned to phenotype group 2. In this group, 23 isolates (96%) belonged to PFGE type D and one isolate to type K. PFGE types A, B, C, D, F, G, K, and N, characterized previously, described epidemic MRSA and MSSA clones, which have been widely disseminated in Poland and globally (Table 1).

As expected, MLVA showed lower discriminatory power than PFGE (25), although by demonstrating good correlation with specific PFGE types assigned to particular phenotype groups, it confirmed the PFGE results. MLVA typing also segregated MRSA from MSSA and hGISA from GSSA isolates (Table 1).

(iii) MLST. MLST was carried out with 37 isolates, including 16 MSSA isolates and 21 MRSA isolates, representing all of the types and subtypes defined by MLVA and PFGE (Tables 1). Seven clonal complexes (CCs) were distinguished among the 110 isolates. CC8 included ST247 and ST239 with its single locus variant, ST241, and a double locus variant, ST254 (13). It was highly prevalent, with 76 isolates in contrast to CC1, CC5, CC15, CC30, CC45, and CC51, which were each represented by a single isolate. The remaining six STs did not belong to any CC and were considered singletons. CC8 was characteristic mostly for isolates from phenotypic groups 1 and 2 (41 and 31 isolates, respectively). Similarly to PFGE and MLVA results, group 3 was much more clonally diverse, with isolates belonging to all seven CCs. All of the hGISA/hTISA isolates belonged to CC8.

(iv) SCC*mec* types. SCC*mec* types (Table 1) were determined for all of the 77 MRSA isolates, and 3 of the 5 different types of SCC*mec* cassette were found in this study (I, III, and IV) (32). SCC*mec* types IA ($n = 33$) and IIIC ($n = 29$) were predominant, while types I ($n = 1$), III ($n = 8$), IIIB ($n = 2$), and IV ($n = 4$) were less common. Most isolates from group 1 harbored SCC*mec* III or one of its previously described variants, IIIB or IIIC (1, 32). SCC*mec* IA was identified for all but one MRSA isolate from the phenotype group 2.

(v) *agr* types. Four *agr* groups (alleles), designated I to IV, were found among all the isolates tested (Table 1). *agr* group I was found among isolates of CC8 and C45. The presence of *agr* group I was also found for a large number of isolates of phenotype groups 1 ($n = 47$) and 2 ($n = 32$). *agr* group II included all isolates belonging to CC15 and CC5, whereas *agr* group III contained all isolates belonging to CC30 and CC1. In *agr* groups I to III, single isolates were found that belonged to completely different genetic backgrounds than those described above. All of the hGISA/hTISA isolates belonged to *agr* group I.

DISCUSSION

The occurrence of species-specific *S. aureus* phenotypic deficiencies can cause difficulties in routine laboratory diagnosis of *S. aureus*, resulting in inappropriate therapy and infection control procedures. The lack of two main species-specific proteins as observed by phenotypic detection methods can lead to misidentification of *S. aureus* isolates. The incidence of coagulase- or CF-deficient strains is estimated to be between 1 and 20% or between 5 and 15%, respectively, of all *S. aureus* isolates (53). The data presented here confirm these findings, with approximately 4.5% ($n = 110$) of all *S. aureus* isolates ($n = 2{,}443$) found not to produce one of the species-specific proteins. Previous Polish studies (45) showed that the appearance of strains deficient in both coagulase and CF simultaneously is as rare as 1%, and in this study such isolates were not been observed. Therefore, *S. aureus* species identification requires detection of both of these proteins during the diagnostic process. The commercially available agglutination kits mainly detect protein A (present in approximately 90% of strains) and CF (53), which, as shown by Blake and Metcalfe, can be hidden by staphylococcal capsule (5). Therefore, in order to improve accuracy of detection, some manufacturers have attached antibodies against staphylococcal capsule types 5 and 8, which account for about 70 to 80% of clinical isolates of *S. aureus* (53). According to previous studies, none of the commercially available tests correctly identified all investigated isolates of *S. aureus* and other staphylococcal species (34). However, the use of anticapsular antibodies improved the detection of *S. aureus* isolates phenotypically deficient in CF and protein A, but the incidence of false positives could increase because some other staphylococcal species also produce type 5 and 8 capsular polysaccharides (5, 53). The phenotypic and genetic characterization of atypical *S. aureus* strains performed in this study allows us to conclude that besides the phenotypic lack of coagulase or CF, these strains often show other unusual characteristics. The most important from a therapeutic point of view is decreased susceptibility to glycopeptides (hGISA/hTISA) in the group of CF-deficient isolates which was first observed in our study. Another interesting observation was the association between the coagulase-deficient phenotype and the lack of thermonuclease. Besides CF masking by staphylococcal capsule (5), there have been several explanations for the CF- or coagulase-deficient phenotype, such as the insertion of a transposon in the *clfA*, *clfB*, or *coa* genes (16, 28, 50), drug-related point mutations in these genes (10), or lysogenic conversion with LS1 and LS2 phages (11). Savolainen et al. (41) and Juuti et al. (21) revealed that some MRSA strains expressed a surface protein, called Pls (plasmin-sensitive protein), that masked the ClfA protein and was responsible for its poor detection in most of the Pls-positive MRSA isolates. Our investigation revealed that the CF-deficient strains studied were in fact MRSA and that they harbored the SCC*mec* type IA, which contained the *pls* gene as described previously (41). Some studies proposed that the *fbpA* gene, encoding fibronectin-binding protein (FnBP) A, and *coa* could be allelic variants of the same gene (43).

We observed three phenotypically deficient groups, and in two of these, phenotype groups 1 and 2, the majority of isolates were clonally related. Most isolates of these two groups belonged to the international Hungarian and Iberian clones, respectively, both of which have been shown to be widely disseminated in Poland and Europe. Phenotype group 3 was found to be much more clonally diverse. These data raise the following question: did phenotypic deficiency in CF or coagulase appear individually, being favorable for single organisms, which then disseminated within the two clones, or did they appear by multiple independent events within both of them? With the data collected in this work, we cannot answer the question unambiguously. The simultaneous appearance of two types of deficiencies in two different genetic backgrounds might be indicative for the first possibility; however, the significant level of diversity of the CF- and coagulase-deficient isolates does not allow elimination of the second one. In the population investigated in our study, 66% of coagulase-deficient and 81% of CF-deficient isolates belonged to one of the major CCs. This is in accordance with previously reported data that revealed the presence of five major evolutionary lineages of *S. aureus*, reflected by five major CCs, into which the majority of epidemic MRSA strains may be classified (CC5, CC8, CC22, CC30, and CC45) (23, 36).

The fact that all investigated strains harbored the *clfA*, *clfB*, *coa*, *spa*, and *nuc* genes, but the presence of the corresponding products was not detected by the phenotypic methods, may suggest the involvement of global regulators in this phenomenon, because they coordinate the expression of a wide array of extracellular and cell wall-associated virulence determinants (39). To date, the best characterized are the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sarA*). The *agr* and *sarA* loci can both directly and indirectly up- and downregulate some genes. In this study, *agr* group I was usually characteristic for two different genetic backgrounds, CC8 and CC45. *agr* group IV was represented by only two isolates with ST121 (CC51).

Speculation that deficiency in CF and the coagulase-deficient phenotype appeared independently in two previously existing clones may also be confirmed by the simultaneous occurrence of the CF-deficient phenotype and decreased susceptibility to glycopeptides (hGISA). This phenomenon was observed only for two isolates belonging to phenotype group 1 [CF(+), COA(−)]. In CF-deficient hGISA strains, the loss of delta-hemolysin production was observed. Sakoulas et al. suggested that hGISA and GISA strains differed from the genetically related vancomycin-susceptible strains by a nonsense mutation in *agrA*, which encodes the transcriptional regulator for the *agr* locus. The expected loss of function conferred by this mutation is consistent with the loss of delta-hemolysin production by GISA strains. Delta-hemolysin is unique among the

secreted virulence factors regulated by *agr*, because it is encoded by the *hld* gene and is derived from translation of RNAIII, the effector molecule of *agr*. Sakoulas et al. raised the question of whether hGISA and GISA strains could belong to *agr* group II, which is predominant in locations other than the United States and Japan (39). However, this was not confirmed in later studies undertaken by Verdier et al. (51), who showed that almost all of the investigated European hGISA and GISA strains belonged to either *agr* group I or II. In our study, all of the hGISA/hTISA isolates were assigned to *agr* group I. We carried out a specific CAMP test (15, 40), with which we noted that hGISA/hTISA strains did not show production of delta-hemolysin. It has been postulated that the lack of delta-hemolysin expression is due to the loss of the *agr* function (38). Data from a British study have shown that hGISA arose in the preexisting clonal complexes CC5, CC8, CC22, CC30, and CC45 (20) but only in strains belonging to *agr* groups I and II. Our study supports this, finding that all strains with reduced susceptibility to glycopeptides belong to CC8 (the Iberian and Hungarian clones) and *agr* group I.

The global regulator *agr* positively regulates expression of many murein hydrolases, including the *atl* gene, which encodes autolysin. An obvious association between lowered *atl* expression and the hGISA/GISA phenotype was observed. *atl* expression was reduced in the GSSA parent strain compared with that of other GSSA isolates not having related hGISA isolates (54). The lower *atl* expression exhibited by the GSSA parent strain suggested that a reduction in *atl* expression may take place prior to the development of glycopeptide-intermediate resistance and that this could therefore be a predisposing factor for the development of the hGISA and GISA phenotype. Autolysin plays a fundamental role in cell division, and its reduced expression may result in the accumulation of peptidoglycan layers and thus a thickened cell wall. Because the expression of autolysin is positively regulated by *agr*, point mutations in the *agr* locus postulated by Sakoulas et al. can result in the altered expression of *atl* and other genes encoding murein hydrolases, which can simultaneously have an influence on the development of reduced susceptibility to glycopeptides. Point mutations, which could occur in the *agr* locus in the case of hGISA strains, could alter other regulators, as well as the expression of the genes encoding surface proteins. Indirect evidence that expression of CF may be altered by acquisition of glycopeptide resistance came from studies carried out by Renzoni et al. (35), who reported that acquisition of glycopeptide (teicoplanin) resistance may alter the levels of expression and surface display of FnBPs in stable TISA compared with its teicoplanin-susceptible parent. FnBPs, like CF, belong to the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family of proteins. However, we postulate that it is also possible that surface proteins can be trapped in the thickened cell wall of some hGISA/GISA strains.

We propose that the coagulase- and thermonuclease-deficient phenotypes were acquired by two widely disseminated *S. aureus* clones. Difficulties in diagnosis of both phenotypic deficiencies and the multiresistance of both clones could be responsible for their ecological success. Data presented in this study demonstrate that the association of the coagulase- and thermonuclease-deficient phenotypes, as well as of CF deficiency and the hGISA phenotype, might be connected with the global regulation system. The high frequency of the simultaneous occurrence of CF deficiency and the hGISA phenotype among MRSA isolates seems to be a cause of concern, particularly since the first signs of reduced susceptibility to glycopeptides usually remain unnoticed. Since only a small subpopulation of isolates is able to grow at higher concentrations of a glycopeptide, the number of cells analyzed in standard susceptibility tests may be too small to ensure the inclusion of this subpopulation. It is generally thought that virulence and decreased susceptibility to glycopeptides in *S. aureus* are the result of a delicate balance in the expression of several genes. It is also clear that the background of the strain influences the in vitro phenotype.

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