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NOTES

Loss of the *mecA* Gene during Storage of Methicillin-Resistant *Staphylococcus aureus* Strains

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The *mecA* gene was lost in 36 (14.4%) of 250 methicillin-resistant *Staphylococcus aureus* isolates after 2 years of storage at −80°C with the Microbank system (Pro-lab Diagnostics, Austin, Tex.). Further analysis of 35 of these isolates confirmed loss of the *mecA* gene in 32 isolates. This finding has important implications for the management of strain collections.

The evaluation of new diagnostic or in vitro antimicrobial susceptibility tests for methicillin-resistant *Staphylococcus aureus* (MRSA) requires well-defined strain collections. In The Netherlands all MRSA strains that have been isolated since 1989 are stored at the National Institute of Public Health and the Environment (RIVM; Bilthoven, The Netherlands). In 1999 we evaluated the MRSA Screen latex agglutination test (Denka Seiken, Ltd.) by the use of a part of the RIVM collection (14). Thereafter, part of this collection was stored at the microbiology laboratory of the Amphia Hospital in Breda, The Netherlands. In 2001 we retested 250 of the MRSA isolates stored at the Amphia Hospital for methicillin resistance. Surprisingly, 36 (14.4%) of the 250 isolates no longer harbored the *mecA* gene.

In the present report we describe a further analysis of 35 of the 36 strains that apparently lost the *mecA* gene. We compared the isolates stored at the Amphia Hospital with renewed subcultures from the original isolates stored at the RIVM.

The 35 MRSA isolates are a subset of a collection of 250 MRSA isolates, comprising 247 different phage types and three isolates that were not typeable, collected in The Netherlands between 1989 and 1998. The original isolates were stored at the RIVM (site B) at room temperature in Moeller agar medium in the year they were isolated. In 1999, subcultures from the original isolates were made at site B and *S. aureus* identification and methicillin resistance determination were performed by multiplex PCR for the coagulase and *mecA* genes as described previously (4, 14). Thereafter, the isolates were transported to and stored at the laboratory of the Amphia Hospital in Breda, The Netherlands (site A), in the commercial Microbank Bacterial Preservation system (Pro-lab Diagnostics, Austin, Tex.). Each Microbank vial contains cryopreservative and approximately 25 porous beads that serve as carriers to support microorganisms. Vials were inoculated according to the manufacturer's instructions. Vials were kept in a −80°C freezer. This freezer is equipped with a sound alarm, which goes off when the temperature drops, to ensure storage conditions. In the summer of 2002, renewed subcultures from the 35 original isolates were requested from site B for further analysis.

The 35 isolates from site A and site B were compared by pulsed-field gel electrophoresis (PFGE). PFGE was performed as described by Murchan et al. (11).

On the isolates from both sites, oxacillin disk diffusion testing according to NCCLS standards using 1-μg oxacillin disks (12), *mecA* gene detection by Southern blotting and hybridization, and multiplex PCR for staphylococcal cassette chromosome *mec* (SCC*mec*) typing were performed.

***mecA* gene detection by Southern blotting.** PFGE gels were blotted onto Hybond N^+ membranes (Amersham, Roosendaal, The Netherlands). Membranes were probed with a PCR-amplified *mecA* gene DNA fragment with the chemiluminescence system provided by Boehringer (Boehringer, Mannheim, Germany). Hybridization was visualized by exposure of the blots to photographic films for various time periods.

Multiplex PCR for *mec* element type assignment was performed as described previously by Oliveira and de Lencastre (13). PCR-amplified material was identified by agarose gel electrophoresis.

PCR for the *mecA* gene and *nuc* gene was performed directly on a bead from the 35 Microbank vials. A single bead was incubated in 100 μl of Tris-EDTA glucose buffer (pH 8.0) containing 1 mg of lysostaphin/ml. After removal of the bead, DNA was extracted according to the protocol published by Boom et al. (2). DNA was dissolved in 100 μl of 10 mM

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TABLE 1. Number of MRSA isolates that lost the *mecA* gene at site A by year of isolation

Yr of isolation	Total no. of isolates	No. (%) of isolates that lost the *mecA* gene
1989	4	0
1990	17	3 (17.6)
1991	22	4 (18.1)
1992	30	6 (20.0)
1993	46	11 (23.9)
1994	45	5 (11.1)
1995	38	6 (15.8)
1998	48	1 (2.1)

Tris-HCl (pH 8.0), and 5-μl portions were subjected to *mecA* PCR according to the procedure of Murakami et al. (10). The PCR for the *nuc* gene was performed as described previously by Brakstad et al. (3).

Broth enrichment culture. A single bead from a Microbank vial was added to Mueller-Hinton (MH) broth and incubated for 48 h at 35°C; thereafter, the broth was subcultured onto an MH agar plate and an MH agar plate supplemented with 2% NaCl. To facilitate the recognition of *mecA* gene-positive isolates, two 1-μg oxacillin disks were placed on each agar plate. The presence of the *mecA* gene was confirmed by MRSA screen test (bioMérieux, Marcy l'Etoile, France) and PCR for the *mecA* gene as described by Murakami et al. (10).

To determine statistical significance, the Fisher's exact test was used. Statistical significance was accepted when the *P* value was <0.05.

Table 1 shows the numbers and percentage of isolates per year of isolation that lost the *mecA* gene as found in our previous evaluation in 2001. Only 2.1% of the MRSA strains isolated in 1998 lost the *mecA* gene, while the percentages were significantly higher, ranging between 11.1 and 23.9%, among the strains isolated earlier ($P = 0.005$).

By comparing the 35 isolates from site A and the original isolates stored at site B by PFGE, the possibility that there had been a mix-up between MRSA and methicillin-sensitive isolates in the freezer at site A was ruled out: all pairs showed a high degree of similarity (Fig. 1.). Nevertheless, small differences were nearly always present, possibly associated with physical loss of the *mecA*-containing genomic locus. In Fig. 1, band differences between the isogenic pairs are highlighted. Major genetic rearrangements seem to have occurred. When the actual chromosomal differences are assessed, 11 out of 35 pairs cannot be discriminated on the basis of PFGE. For six pairs, the chromosomal size of the isolate from site A, ranging between 10,000 and 450,000 bp, is larger than that of the isolate from site B (Table 2). For the remaining 18 strains, deletions ranging between 10,000 and 350,000 bp can be documented for the isolates from site A (Table 2).

In Fig. 2, the results from our consecutive attempts to detect the *mecA* gene from the isolates stored at site A and site B are presented. In Table 2, the results of the oxacillin disk diffusion, the *mecA* gene detection by Southern blotting, and the SCC*mec* multiplex PCR are presented per isolate. In summary, the *mecA* gene was detected, either by Southern blotting or by SCC*mec* multiplex PCR, in 3 (8.6%) of the 35 isolates from site A and in 14 (40.0%) of the 35 corresponding isolates from site B ($P = 0.004$).

The PCRs for the *nuc* gene and the *mecA* gene that were performed directly on the beads from the Microbank vials detected the *nuc* gene in each of the 35 vials; however, the *mecA* gene could only be detected from beads from two vials. From one of these two vials, one of the SCC*mec*-positive isolates (strain 95-900) was cultured.

Enrichment culture retrieved nine (25.7%) *mecA* gene-positive strains from the 35 Microbank vials. Among these nine strains were the three SCC*mec* PCR-positive strains and the two strains from the Microbank vials in which the *mecA* was detected by PCR directly on a bead. Subculturing the broth onto MH agar (with or without 2% NaCl) onto which 1-μg oxacillin disks were placed yielded similar results, recovering seven and nine strains, respectively. The subculture had to be incubated for at least 48 h. In addition to culturing a bead in a nonselective MH broth, we also cultured in two selective MH broths containing 4 mg of oxacillin/liter and 16 mg of cefoxitin/liter, respectively. This, however, did not result in a higher yield of *mecA* gene-positive isolates; a total of seven *mecA* gene-positive isolates were detected in each of the two selective broths.

Loss of the *mecA* gene in such a large percentage of MRSA isolates during storage at −80°C with the Microbank system has never been described before. Hürlimann-Dalel et al. described the apparent loss of the *mecA* gene in methicillin-resistant *S. aureus* isolates stored as lyophilized cultures (7). However, they did not confirm the presence of the *mecA* gene at the time the isolates were stored; therefore, it is not certain that all isolates carried the *mecA* gene to start with (7). Loss of the *mecA* gene has also been observed in vivo (5, 9). Katayama et al. have demonstrated that the SCC*mec*, which contains the *mecA* gene, can be integrated to and excised from the *S. aureus* chromosome (8). However, spontaneous excision of the SCC*mec* did not occur appreciably in the strain that was examined (8).

The present study confirms the loss of the *mecA* gene in 32 of the 35 strains stored at site A that were examined more closely. In three isolates the *mecA* gene could be detected again without any extra effort. Enrichment culture retrieved *mecA* gene-positive isolates from nine Microbank vials. Of the original 35 strains stored at site B, 21 (60%) had also lost the *mecA* gene. This suggests significant genetic instability in these strains.

Comparison of the isolates stored at site A and at site B by PFGE showed that these isolates are essentially the same isolates, although differences were detected in 24 of the 35 strains. Insertion and deletion events have occurred during storage. The availability of isogenic pairs of strains showing differently sized deletions and insertions allows for detailed examination of the region of excision.

All vials still contained viable *S. aureus* isolates; the result of *nuc* PCR performed directly on a bead from the Microbank system was positive, and cultures yielded *S. aureus*. Surprisingly, the result of PCR for the detection of the *mecA* gene directly on a bead was positive for two vials only. Not only the *mecA* gene-containing strain but also the genetic element itself was lost upon storage at −80°C.

A statistically significantly lower percentage (2.1%) of

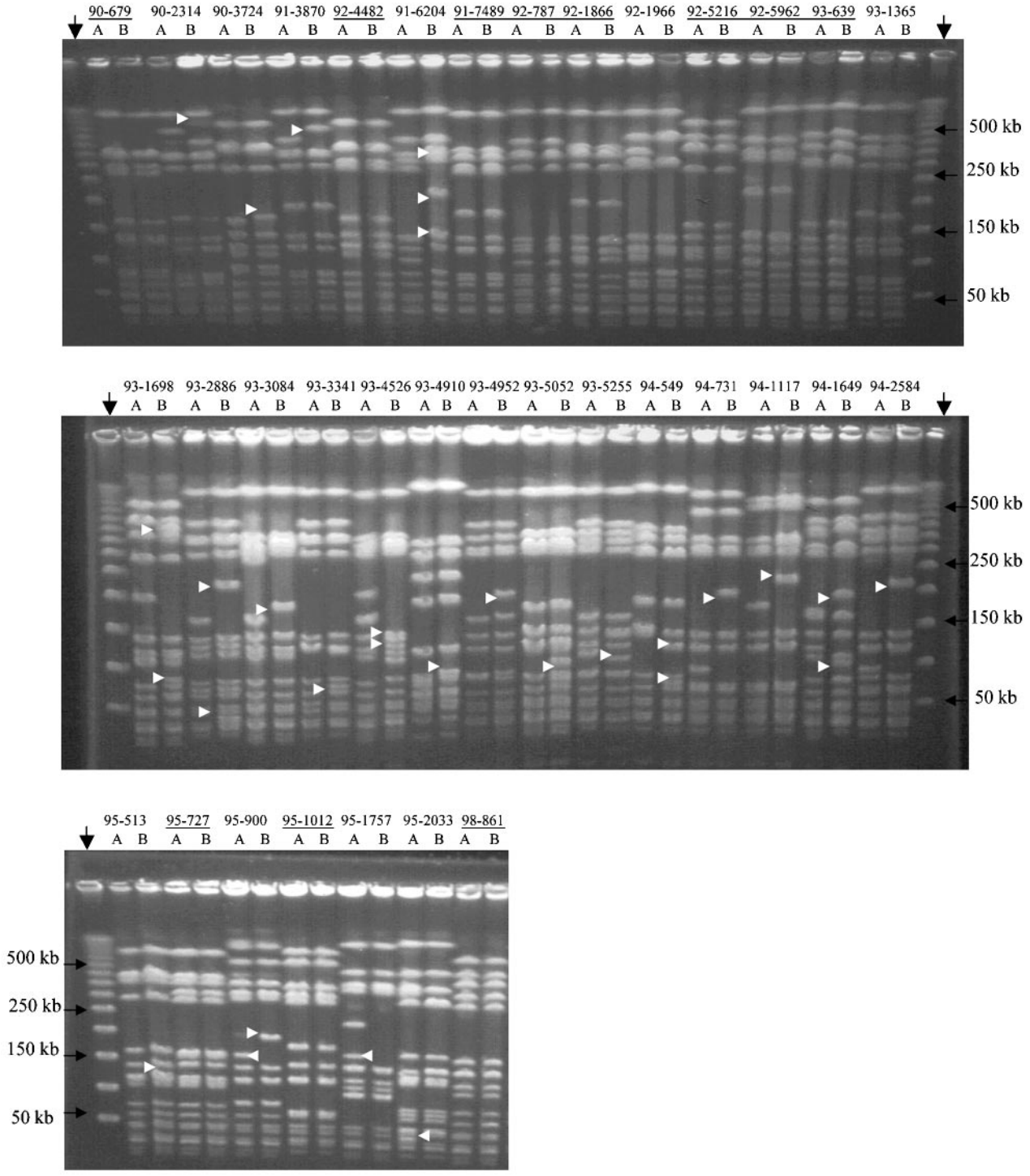

FIG. 1. Genetic comparison of 35 isogenic and presumptively MRSA isolates from site A (Amphia Hospital, Breda, The Netherlands) and site B (RIVM) by pulsed field gel electrophoresis. Strain identification codes are given above the lanes; codes are underlined if the paired isolates are identical. White arrowheads highlight major differences in banding patterns observed between the isogenic pairs. The vertical arrows above the lanes identify the marker lanes containing concatemeric bacteriophage lambda DNA. The sizes of four of the marker segments are given next to the panels in kilobase pairs (kb), indicated by horizontal black arrows.

TABLE 2. Results of the tests comparing subcultures on blood agar from stored MRSA strains[a]

Strain	Site A				Site B				Genomic changes[e]
	Oxacillin disk diffusion[b]		*mecA* gene[d]	SCC*mec* type	Oxacillin disk diffusion[b]		*mecA* gene[d]	SCC*mec* type	
	Zone diam (mm)	Result[c]			Zone diam (mm)	Result[c]			
90-679	19	S	N		14	S	N		ND
90-2314	21	S	N		21	S	N		D 50,000
90-3724	18	S	N		14	S	N		D 10,000
91-3870	20	S	N		22	S	N		D 50,000
91-4482	18	S	N		13	S	N		ND
91-6204	26	S	N		0	R	P	IV	D 350,000
91-7489	24	S	N		24	S	N		ND
92-787	18	S	N		19	S	N		ND
92-1866	17	S	N		0	R	N	III	ND
92-1966	18	S	N		18	S	N		i 450,000
92-5216	17	S	N		0	R	N	III	ND
92-5962	16	S	N		0	R	P	III	ND
93-639	17	S	N		17	S	N		ND
93-1365	23	S	N		20	S	N		i 120,000
93-1689	18	S	N		17	S	N		D 330,000
93-2886	0	R	N	I	0	R	P	I	D 50,000
93-3084	15	S	N		15	S	N	III	D 20,000
93-3341	16	S	N		22	S	N		D 70,000
93-4526	19	S	N		0	R	P	III	i 100,000
93-4910	18	S	N	IV	22	S	N		D 80,000
93-4952	15	S	N		0	R	P	III	D 200,000
93-5052	14	S	N		17	S	N		D 10,000
93-5255	17	S	N		0	R	P		i 10,000
94-549	23	S	N		24	S	N		D 200,000
94-731	19	S	N		0	R	P	IV	D 100,000
94-1117	23	S	N		0	R	P	IV	D 50,000
94-1649	12	S	N		16	S	N		D 180,000
94-2584	21	S	N		0	R	P	IV	D 120,000
95-513	13	S	N		19	S	N		D 120,000
95-727	18	S	N		18	S	N		ND
95-900	0	R	N	III	0	R	P	III	D 40,000
95-1012	17	S	N		23	S	N		ND
95-1757	15	S	N		15	S	N	III	i 350,000
95-2033	17	S	N		19	S	N		i 10,000
98-861	14	S	N		14	S	N		ND

[a] Storage at site A in Microbank system at −80°C since 1999; storage at site B since the year of isolation (1990 to 1998) in Moeller agar medium at room temperature.
[b] Oxacillin disk diffusion according to NCCLS standards.
[c] R, resistant; S, susceptible.
[d] N, negative result; P, positive result.
[e] i, insertion in strain from site A ($n = 6$) in base pairs; D, deletion in strain from site A ($n = 18$). in basepairs; ND, no difference ($n = 11$).

strains collected in 1998 lost the *mecA* gene compared to the results seen with strains collected in earlier years. It can be hypothesized that MRSA strains consist of a heterogeneous population of both *mecA*-positive and *mecA*-negative cells. These *mecA* gene-negative cells initially comprise only a small minority of the population. If the *mecA* gene-negative cells better resist the storage conditions, they have a selective advantage; the longer the storage period, the larger the effect of this selective advantage and, hence, the larger the effect of longer storage periods on the loss of *mecA*-positive cells. The fact that in 1999 all isolates, irrespective of their year of isolation, were *mecA* positive seems to refute our hypothesis. However, when they were stored at −80°C in 1999, the older strains could have contained a larger percentage of *mecA*-negative cells to begin with, due to their longer storage period, than did the more recently isolated strains.

One of the explanations for the different numbers of *mecA* gene-positive isolates detected in the study in 2001 and in the present study might be that each time, another single bead from the Microbank vial was used. Each Microbank vial contains approximately 25 beads. Probably, not all the beads are coated with identical amounts of *mecA* gene-positive cells. This agrees with our hypothesis that each MRSA strain consists of a heterogeneous population of cells containing both *mecA* gene-positive and -negative cells in the first place.

Preservation of strains in a microbiology laboratory is of great importance for quality control, teaching, and research (6). Freezing is a very common method of preservation and storage of microorganisms (1). Studies concentrate on the viability of the microorganisms after a certain storage period. Little attention is given to the influence of storage conditions on characteristics of the stored strain such as antimicrobial susceptibility. The Microbank Bacterial Preservation system (Pro-lab Diagnostics) is a well-known system for freezer storage of all kinds of microorganisms and is used in laboratories all over the world. At the laboratory of the RIVM (site B), MRSA isolates are stored at room temperature in Moeller agar medium. Statistically significantly more of the isolates

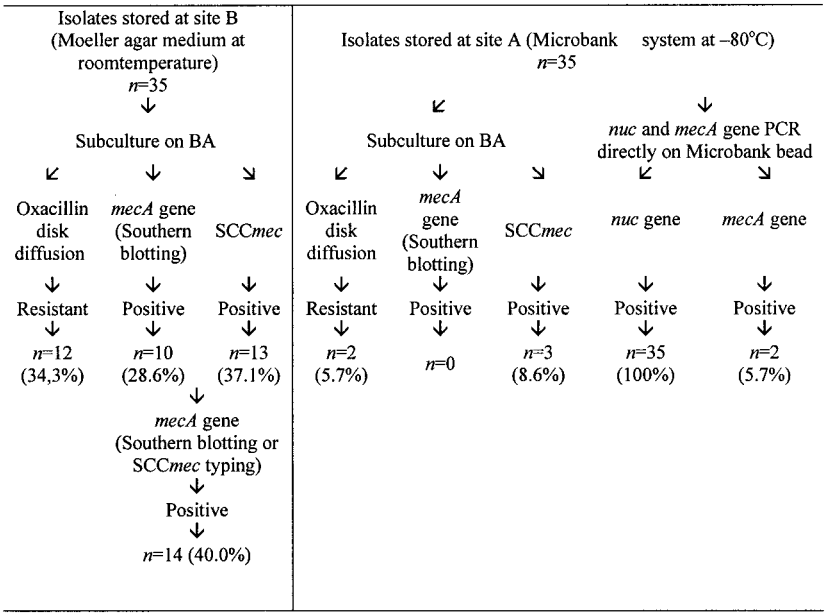

FIG. 2. Results of the experiments performed in 2002 to detect the *mecA* gene in the isolates stored at site A and site B. Oxacillin disk diffusion assays were performed according to NCCLS standards. BA, blood agar.

from site B (40%) contained the *mecA* gene than did the isolates stored at site A (8.6%; $P = 0.004$). In this study we concentrated on 35 isolates of a collection that consists of 250 MRSA isolates. We do not know what happened to the *mecA* gene in the other isolates. Therefore, we cannot conclude that the *mecA* gene was lost more frequently at site A. One of the issues that remain is whether loss of the *mecA* gene is related to the storage method.

This study clearly demonstrates that *mecA* can be lost from MRSA strains stored at −80°C with the Microbank system. This has important implications for the management of strain collections. Prior to the use of MRSA isolates that have been previously stored at −80°C in any study, they have to be checked for the presence of the *mecA* gene at that moment in time. Maybe storage of MRSA strains can be improved by altering the storage conditions by, for example, the addition of oxacillin to the cryopreservative. This needs to be evaluated in future studies.

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