ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2005, p. 1216–1219 Vol. 49, No. 3
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.3.1216–1219.2005
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mecA Is Not Involved in the σ^B-Dependent Switch of the Expression Phenotype of Methicillin Resistance in *Staphylococcus epidermidis*

Johannes K.-M. Knobloch,* Sebastian Jäger, Jörn Huck, Matthias A. Horstkotte, and Dietrich Mack†

Universitätsklinikum Hamburg-Eppendorf, Zentrum für Klinisch-Theoretische Medizin, Institut für Infektionsmedizin, Hamburg, Germany

Received 21 May 2004/Returned for modification 11 August 2004/Accepted 7 November 2004

A lack of σ^B activity reduces methicillin resistance in heterogeneous *Staphylococcus epidermidis* 1057, whereas inactivation of the anti-sigma factor RsbW switched the phenotype to homogeneous expression of resistance. Oxacillin induction of *mecA* transcription is reduced in a σ^B-negative strain. However, *mecA* is not involved in the switch of expression phenotype.

Staphylococcus epidermidis is the predominant cause of foreign-body-associated infections (19, 21). A major problem with these organisms is the widespread methicillin resistance of clinical isolates (1, 2, 5), which is often linked to the presence of the *icaADBC* operon responsible for biofilm formation in *S. epidermidis* (3, 4). Additionally, a population of attached *S. epidermidis* single cells is able to persist even under extremely high antibiotic concentrations, as demonstrated for methicillin-resistant *S. epidermidis* 1057 (10).

Recently, we demonstrated that the inactivation of *rsbU* (encoding a positive regulator of the alternative sigma factor σ^B) reduced methicillin resistance and decreased biofilm formation in *S. epidermidis* (6, 13). In methicillin-resistant *Staphylococcus aureus* (MRSA), it was shown that σ^B activity is required for the expression of high-level methicillin resistance (23), indicating a similar regulation. Interestingly, the role of σ^B in the regulation of biofilm formation in *S. aureus* seems to be different from that in *S. epidermidis*. Recently, Valle et al. (22) demonstrated that the regulatory protein SarA and not σ^B is essential for biofilm formation in *S. aureus*, whereas an influence of σ^B could be observed by Rachid et al. only under osmotic stress conditions for a single isolate of a collection of *S. aureus* strains (17). Additionally, the environmental conditions required for biofilm formation by *S. aureus* differ significantly from those required by *S. epidermidis* (8).

In staphylococci, methicillin resistance as well as biofilm formation are influenced by a variety of environmental factors, such as available nutrients and increased osmolarity or the presence of antibiotics (7, 8, 12, 18). By generation of *S. epidermidis* 1457 mutants with a deletion of the σ^B operon, we could demonstrate that the *rsbU*-dependent regulation of biofilm formation is mediated by the regulation of σ^B activity (9). To investigate the influence of σ^B activity on methicillin resistance in *S. epidermidis*, we transduced the deletions of the σ^B operon of *S. epidermidis* in this study from the methicillin-susceptible genetic background of *S. epidermidis* 1457 (Table 1) into heterogeneous methicillin- and penicillin-resistant *S. epidermidis* strain 1057 as described previously (9), resulting in the *rsbU*, *rsbV*, *rsbW*, *sigB*, *rsbUVW*, and *rsbUVWsigB* mutants (Table 1). In order to specify the structural type of the SCC*mec* cassette of *S. epidermidis* 1057, we performed a multiplex PCR assay (16). Except for the fragment specific for the *kdp* locus, *S. epidermidis* 1057 displayed the fragment pattern which is characteristic for the type II SCC*mec* cassette (Fig. 1), including the *mecI*-specific fragment. Additionally, the *mecR* gene could be detected by PCR in *S. epidermidis* 1057 (data not shown). These data indicate that *S. epidermidis* 1057 harbors an intact *mecI*/*mecR* regulatory system.

The expressed phenotype of methicillin resistance by the generated mutants was investigated by population analysis on Mueller-Hinton (MH) agar supplemented with 2% NaCl and increasing concentrations of oxacillin, as described previously (13). Wild-type *S. epidermidis* 1057 displayed heterogeneous expression of methicillin resistance with an already >2-log-fold reduction of growing colonies at an oxacillin concentration of 2.5 μg/ml (Fig. 2), whereas a small subpopulation was able to grow even at 200 μg of oxacillin/ml. Mutants with dysfunctional σ^B activity displayed an even more sensitive distribution of the population, with an additional ~10-fold reduction of growing colonies at oxacillin concentrations between 2.5 and 50 μg/ml. However, a similar fraction of the population was still able to grow at 200 μg of oxacillin/ml (Fig. 2). Interestingly, in mutants with deletions of *rsbW*, the heterogeneous expression phenotype of the wild type was shifted to a homogeneous expression of methicillin resistance, with MICs for about 90% of the population of at least 50 μg/ml (Fig. 2). These data indicate that the alternative sigma factor σ^B is required for the modulation of the phenotypic expression of methicillin resistance in *S. epidermidis*, as was observed in *S. aureus* (23). We could thereby demonstrate for the first time that the inactivation of the negative regulator of σ^B activity RsbW causes the phenotypic switch from a heterogeneous to a homogeneous expression of methicillin resistance. This finding is additionally corroborated by the observation of reduced oxacillin resistance in

* Corresponding author. Mailing address: Universitätsklinikum Hamburg-Eppendorf, Zentrum für Klinisch-Theoretische Medizin, Institut für Infektionsmedizin, Martinistr. 52, D-20246 Hamburg, Germany. Phone: 49 40 42803 3147. Fax: 49 40 42803 4881. E-mail: knobloch@uke.uni-hamburg.de.

† Present address: Medical Microbiology and Infectious Diseases, The Clinical School, University of Wales Swansea, Swansea SA2 8PP, United Kingdom.

TABLE 1. *S. epidermidis* strains used in this study

Strain	Reference or source	Comments
1457	14	*mecA*-negative isolate from infected central venous catheter
1457*rsbU*	9	*rsbU*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement; strongly repressed σ^B activity
1457*rsbV*	9	*rsbV*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement; strongly repressed σ^B activity
1457*rsbW*	9	*rsbW*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement; constitutive σ^B activity
1457*sigB*	9	*sigB*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement; lack of σ^B activity
1457*rsbUVW*	9	*rsbUVW*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement; constitutive σ^B activity; lack of autoinduction from the internal σ^B-dependent promoter
1457*rsbUVWsigB*	9	*rsbUVWsigB*::*erm* derivate from *S. epidermidis* 1457 derived by allelic gene replacement; lack of σ^B activity
1057	15	*mecA*- and *blaZ*-positive isolate from infected central venous catheter
1057*rsbU*	This study	Transductant of *S. epidermidis* 1057 from the 1457*rsbU*
1057*rsbV*	This study	Transductant of *S. epidermidis* 1057 from the 1457*rsbV*
1057*rsbW*	This study	Transductant of *S. epidermidis* 1057 from the 1457*rsbW*
1057*sigB*	This study	Transductant of *S. epidermidis* 1057 from the 1457*sigB*
1057*rsbUVW*	This study	Transductant of *S. epidermidis* 1057 from the 1457*rsbUVWsigB*
1057*rsbUVWsigB*	This study	

the *rsbU* and *rsbV* mutants, in which the activity of the anti-RsbW factor RsbV is suppressed or lacking completely, whereas the *sigB* gene is still intact. These data indicate that the *rsbW* gene could be a locus of so-called *chr*∗ mutations (20), which are responsible for phenotypic switches observed with heterogeneously resistant methicillin-resistant *S. epidermidis* and MRSA strains in vivo during therapy.

For further transcriptional analysis, the *rsbUVWsigB* and *rsbUVW* strains were characterized. To investigate σ^B activity and its relevance for the transcription of *mecA* in these mutants, transcriptional analyses were performed by real-time reverse transcription (RT)-PCR. As a marker for σ^B activity, the *asp23* gene of *S. epidermidis*, which is transcribed from at least two different σ^B-dependent promoters (9), was used. The strains were cultivated prior to RNA extraction in MH broth supplemented with 2% NaCl (MH_{NaCl}) as well as in MH_{NaCl} supplemented with a subinhibitory concentration of 1 μg of oxacillin/ml (MH_{oxa}). In both media, the investigated strains displayed almost identical growth curves, except for the *rsbUVWsigB* strain, which displayed a slight delay of growth (25 to 45 min during exponential growth) in MH_{oxa}. However, the cell densities during stationary phase were almost identical for all strains and media (data not shown). RNA was extracted at a time point when all strains were in the mid-exponential growth phase (9 h in both media) as well as during stationary phase (17 h) by using a modified protocol of the RNeasy bacteria kit (QIAGEN, Hilden, Germany) as described previously (9). The cutoff for significant differences in regulation was defined as 2.5-fold up- or down-regulation of the respective genes (9). RT-PCR was performed in an iCycler thermal cycler using the oligonucleotides *gyrB*-real1 (5′-CTGACAATGGCCGTGGTATTC-3′), *gyrB*-real2 (5′-GAAGATCCAACACCGTGAAGAC-3′), *asp23*-real1 (5′-TCCAACTTCTACAGATACGCC-3′), *asp23*-real2 (5′-AAAATTGCAGGTATTGCAGC-3′), *mecA*-real1 (5′-ATTATGGCTCAGGTACTGCTATC-3′), and *mecA*-real2 (5′-CTGGTGAAGTTGTAATCTGGAAC-3′) as described previously (9). Relative transcriptional levels within distinct experiments were determined by using the $2^{-\Delta\Delta C}{}_T$ method (11) and compared to the wild type with *gyrB* as the reference housekeeping gene. RT-PCR was performed in triplicate in each of three independent experiments. As observed for the *S. epidermidis* 1457 mutants, transcription of the σ^B-dependent gene *asp23* was significantly reduced in the *rsbUVWsigB* strain (Table 2). The observed differences were more pronounced under conditions of oxacillin induction. In the *rsbUVW* mutant, only marginal differences of *asp23* transcription were observed (Table 2). Thereby, in MH_{NaCl}, σ^B activity was increased during exponential phase and decreased during stationary phase, whereas in MH_{oxa}, σ^B activity was decreased during exponential growth phase but similar to that of the wild type in stationary phase. The decrease of σ^B activity in the *rsbUVW* strain compared to the wild type could be explained by the lack of transcription from the internal σ^B-dependent promoter preceding *rsbV* in the σ^B operon of *S. epidermidis* (6) and thereby the lack of σ^B autoinduction under the investigated conditions.

Interestingly, the homogeneously resistant *rsbUVW* mutant

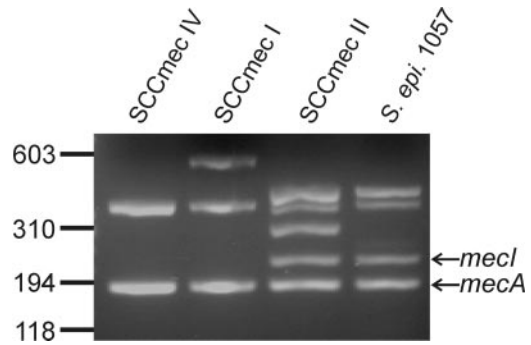

FIG. 1. Characterization of the SCC*mec* cassette in *S. epidermidis* 1057. The SCC*mec* element was characterized by a multiplex PCR (16). Typical representatives of the SCC*mec* types IV, I, and II of MRSA are displayed in lanes 1 to 3. Except for the fragment specific for the *kdp* locus, *S. epidermidis* 1057 displayed the fragment pattern which is characteristic for the type II SCC*mec* cassette. The fragments representing the genes *mecA* and *mecI* are indicated.

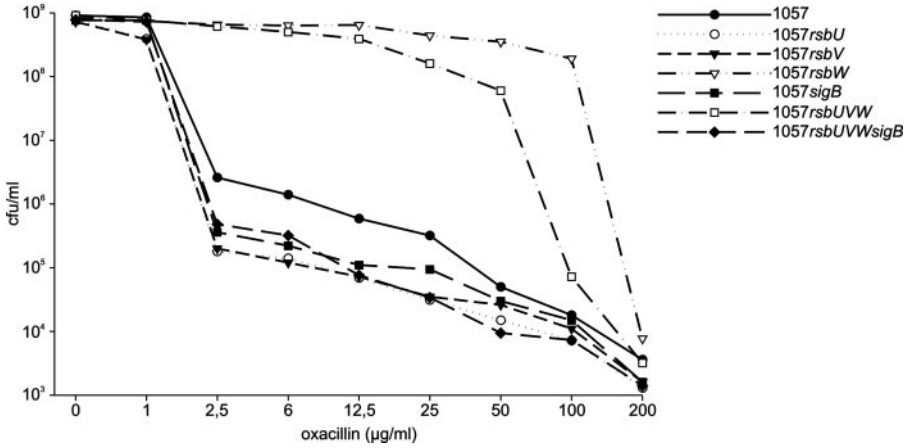

FIG. 2. Phenotypic characterization of methicillin resistance by population analysis. Wild-type *S. epidermidis* 1057 displayed a heterogeneous expression of methicillin resistance, with a more than 2-log-fold reduction at an oxacillin concentration of 2.5 μg/ml. Mutants with dysfunctional σ^B (the *rsbU*, *rsbV*, *sigB*, and *rsbUVWsigB* strains) displayed a more sensitive distribution, with about an additional 1-log-fold reduction of cells at lower oxacillin concentrations. In mutants with inactivation of the anti-sigma factor RsbW (the *rsbW* and *rsbUVW* strains), the heterogeneous expression phenotype of the wild type was shifted to a homogeneous expression of methicillin resistance.

displayed no significant differences from the wild type in *mecA* transcription under all investigated conditions, whereas in the less resistant *rsbUVWsigB* mutant, *mecA* transcription was slightly induced in MH_{NaCl} (Table 2). However, investigating the induction of *mecA* transcription during growth in medium supplemented with a subinhibitory concentration of oxacillin (1 μg/ml) revealed that in the σ^B-negative mutant, the effect of oxacillin induction was smaller than in the wild type and the *rsbUVW* mutant. During exponential growth, oxacillin induction led to 102-fold and 66-fold increases of *mecA* transcription in the wild type and the *rsbUVW* mutant, whereas in the *rsbUVWsigB* mutant, a 25-fold increase was observed. The differences in oxacillin induction were more prominent during stationary phase, in which only a 3-fold induction could be observed for the *rsbUVWsigB* strain, whereas 1057 and the *rsbUVW* strain displayed 27- and 32-fold increases, respectively.

Transcriptional analysis revealed that a lack of σ^B activity leads to a dysfunctional regulation of *mecA* transcription with increased transcriptional activity without oxacillin induction and on the other hand a reduced induction by oxacillin, especially during the stationary phase. Interestingly, despite the phenotypic switch of the *rsbUVW* mutant to a homogeneous expression of oxacillin resistance, no significant differences in *mecA* transcription compared to the heterogeneous wild type could be observed, indicating that besides the expected inducibility by oxacillin, the σ^B regulation of genes other than *mecA* must be responsible for differences in the phenotypic expression of oxacillin resistance in *S. epidermidis*.

TABLE 2. Transcriptional differences between mutants and the wild type

Strain	Gene	Transcriptional difference compared to the wild-type strain[a]			
		MH_{NaCl}		MH_{oxa}	
		Exponential	Stationary	Exponential	Stationary
1057*rsbUVW*	*asp23*	**+3**	**−5**	**−5**	+2
1057*rsbUVW*	*mecA*	+1	+1	−2	+1
1057*rsbUVWsigB*	*asp23*	**−24**	**−42**	**−71**	**−604**
1057*rsbUVWsigB*	*mecA*	**+6**	**+5**	+2	−2

[a] The differences of transcription (*n*-fold) between mutants and wild-type *S. epidermidis* 1057 were calculated by the $2^{-\Delta\Delta C_T}$ method. Values represent the mean of results from three independent experiments. Significant differences are displayed in bold.

We thank Rainer Laufs for his continuous support.

This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Werner-Otto-Stiftung, and the Forschungsförderungsfonds Medizin des Universitätsklinikums Hamburg-Eppendorf, Hamburg, Germany, given to J.K.-M.K. and D.M.

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