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FEMS Microbiology Letters 153 (1997) 261–264

FEMS MICROBIOLOGY LETTERS

Lif, the lysostaphin immunity factor, complements FemB in staphylococcal peptidoglycan interpeptide bridge formation

M. Tschierske [a], K. Ehlert [b], A.M. Strandén [a], B. Berger-Bächi [a,*]

[a] *Institute of Medical Microbiology, University of Zürich, Gloriastr. 32, CH-8028 Zürich, Switzerland*
[b] *Bayer AG, PH-Research Antiinfectives I, D-42096 Wuppertal, Germany*

Received 9 May 1997; accepted 15 May 1997

Abstract

The formation of the *Staphylococcus aureus* peptidoglycan pentaglycine interpeptide chain needs FemA and FemB for the incorporation of glycines Gly2-Gly3, and Gly4-Gly5, respectively. The lysostaphin immunity factor Lif was able to complement FemB, as could be shown by serine incorporation and by an increase in lysostaphin resistance in the wild-type as well as in a *femB* mutant. However, Lif could not substitute for FemA in *femA* or in *femAB*-null mutants. Methicillin resistance, which is dependent on functional FemA and FemB, was not complemented by Lif, suggesting that serine-substituted side chains are a lesser substrate for penicillin-binding protein PBP2′ in methicillin resistance.

Keywords: *Staphylococcus aureus*; Peptidoglycan; Lysostaphin immunity factor; Methicillin resistance

1. Introduction

A characteristic property of the *Staphylococcus aureus* peptidoglycan is the long and flexible pentaglycine interpeptide bridge that allows a high cross-linking of the peptidoglycan strands [1]. It also acts as an anchor for cell wall directed surface proteins [2]. The interpeptide bridge is synthesized by sequential addition of glycine residues by cell wall specific glycyl-tRNA [3], and requires the action of at least two proteins, FemA and FemB, that are encoded by the *femAB* operon. FemA directs the incorporation of the glycines Gly2-Gly3, FemB that of the glycines Gly4-Gly5 [4]. *femAB*-like sequences could be detected by Southern blots in all staphylococcal type strains (unpublished results) and a *femAB*-like operon was characterized in *S. epidermidis* [5] suggesting that FemA and FemB analogues belong to staphylococcal housekeeping genes needed in the biosynthesis of the interpeptide bridge.

FemA and FemB show similarity to the lysostaphin immunity factor Lif [6]. Lif mediates resistance to lysostaphin, a glycylglycine endopeptidase that seems to cleave preferentially between the third and the fourth glycine residues of the pentaglycine cross-bridge. By increasing the serine content and reducing the glycine content of the staphylococcal peptidoglycan in *S. simulans*, *S. carnosus* [6] and *S. aureus* [7] Lif creates bonds unable to be hydrolyzed by lysostaphin. A further glycyl-glycine endopeptidase has been isolated from *S. capitis* [8], suggesting that this resistance mechanism may be of use in other staphylococcal species. Interestingly, a lysostaphin-like en-

* Corresponding author. Tel.: +41 (1) 634 26 50; Fax: +41 (1) 634 49 06; E-mail: bberger@immv.unizh.ch

PII S0378-1097(97)00234-6

zyme has been isolated from an unrelated organism, namely *Streptococcus zooepidemicus* [9], where resistance to this enzyme may be postulated to function by a similar mechanism. FemAB-like analogues may be not restricted to staphylococci.

In *S. aureus*, the pentaglycine side chain plays an important role in methicillin resistance. It is the substrate for the additional penicillin-binding protein PBP2′, a transpeptidase with low affinity to methicillin that in the presence of β-lactam antibiotics takes over the function of the cells' own susceptible PBPs in cell wall biosynthesis [10]. Shortening of the pentaglycine side chain, as a result of inactivation of FemA or FemB, abolishes methicillin resistance and leads to β-lactam hypersusceptibility [4]. We therefore investigated whether Lif may complement the *S. aureus* FemA and/or FemB mutants by inserting serine for glycine in the side chain, and if, by restoring the full length of the side chain, methicillin resistance can be expressed again.

2. Materials and methods

2.1. Strains, growth conditions and resistance tests

The strains are all derived from *S. aureus* NCTC8325. The heterogeneously methicillin-resistant strain BB270 (*mec*) [4] is the parent of the methicillin-susceptible *femB* mutant BB815 (*mec*, Ω2006*femB*::Tn*551*) [11], the *femA* mutant UK17 (*mec*, ochre mutation in *femA*) [12], and the *femAB* null mutant AS145 (*mec*, Δ*femAB*::*tetK*) [4]. Plasmid pCXlif contained the *lif* gene under control of the xylose-inducible promoter of *xylA* [6]. Plasmid pCX26Δlip [13] with the same promoter was used for negative controls. Both plasmids were kindly provided by G. Thumm, Tübingen. Strains were grown in LB broth (Difco, Detroit, MI) at 37°C. Where appropriate, chloramphenicol was added (20 μg ml^{-1}). For xylose-induced expression of *lif*, 0.5% of an overnight culture was used to inoculate the main culture containing 1.0% xylose for induction of *lif*. Incubation was for 7–10 h. Resistance tests were done according to the recommendations of the NCCLS [14]. Minimal inhibitory concentrations (MIC) of methicillin were determined with E strips (Solna, Sweden) [15] on LB plates containing 1.0% xylose and chloramphenicol where needed. The MIC of lysostaphin (AMBI, Trowbridge, UK) was determined by microbroth dilution at 35°C in LB broth supplemented with 1.0% xylose and chloramphenicol where required.

2.2. Plasmid preparation, DNA transduction

Staphylococcal plasmid DNA was prepared using NucleoSpin columns (Machery-Nagel AG, Oensingen) according to the manufacturer's instructions except that the cells were incubated for 10 min at 37°C in buffer A1 containing lysostaphin (50 μg ml^{-1}) before buffer A2 was added. Transduction of the plasmids into *S. aureus* was done by phage 80α with selection of transductants in the presence of chloramphenicol (20 μg ml^{-1}) [4].

2.3. Cell wall composition

For the preparation of peptidoglycan and amino acid analysis the protocols described earlier were followed, except that the cells were grown in LB broth instead of brain heart infusion medium, supplemented with chloramphenicol (20 μg ml^{-1}) and 1.0% xylose where required [4].

3. Results

The plasmid pCXlif, containing the *lif* gene under the control of a xylose promoter, and control plasmid pCX26Δlip were transduced into the methicillin-resistant control strain BB270, into *femA* mutant UK17, *femB* mutant BB815 and *femAB* null mutant AS145. We were repeatedly unable to transduce the control plasmid pCX26Δlip into AS145. Even lowering the concentration of chloramphenicol for selection to 5 μg ml^{-1} did not yield transductants. For unknown reasons, transduction frequencies in AS145 were generally 10^3 times lower than in BB270.

To determine whether Lif was able to complement FemA or FemB or both, the amino acid composition of the purified peptidoglycan obtained after xylose-mediated *lif* induction in the transductants was analyzed and compared to that of the original uncomplemented recipients. Table 1 shows clearly that the serine content increased to 0.35 and more in Mc^r

strain BB270/pCXlif and in the *femB* mutant BB815/pCXlif, whereas in the *femAB* null mutant AS145/pCXlif and the *femA* mutant UK17/pCXlif it remained low. The apparent fourfold serine increase in AS145/pCXlif compared to AS145 is below the experimental reproducible values and therefore not significant. Lif seems to be able to promote serine incorporation into positions 4 and/or 5 of the interpeptide side chain when glycines-2 and -3 are present, but not when Gly2-Gly3 are missing as in the latter two mutants which lack FemA. This points to a FemB-like function of Lif. The serine plus glycine values were not restored to wild-type levels in the *femB* mutant by Lif, suggesting that Lif functions suboptimally in *S. aureus* and will not complement each side chain.

The pentaglycine side chain is the substrate for lysostaphin, a glycyl-glycine endopeptidase which is thought to cleave between Gly3 and Gly4 [2]. The *fem* mutants containing only one glycine in the side chain, namely UK17 and AS145, were resistant to lysostaphin as expected (Table 2). Interestingly BB815, which has a triglycine chain, was still as susceptible as the wild-type BB270. When, however, Lif was expressed in the latter two strains, lysostaphin resistance increased significantly, whereas the control plasmid had no influence on lysostaphin resistance.

Methicillin resistance in staphylococci is only expressed in strains with functional FemA and FemB. Any shortening of the side chain results in loss of resistance. Since all strains carried the *mec* determinant, we wondered if complementation of FemB by Lif would restore methicillin resistance. Although Lif inserted serine residues, it did not increase methicillin resistance as can be seen in Table 2. Growth of these strains on plates containing a methicillin gradient, a more sensitive method to test relative differences in resistance levels, confirmed these results (data not shown).

Table 1
Amino acid analysis of the purified peptidoglycans (molar mass ratio relative to Glu)

Strain	Glu	Ser	Gly	Ala	Lys	Ser+Gly
BB270	1.00	0.05	4.27	2.43	0.83	4.32
BB270/pCXlif	1.00	0.35	3.56	1.80	0.88	3.91
BB815	1.00	0.09	2.67	1.88	0.91	2.76
BB815/pCXlif	1.00	0.37	2.29	1.85	0.88	2.66
UK17	1.00	0.06	0.97	2.30	0.83	1.03
UK17/pCXlif	1.00	0.08	1.00	2.13	0.96	1.08
AS145	1.00	0.02	0.94	2.11	0.86	0.96
AS145/pCXlif	1.00	0.08	0.91	1.99	0.91	0.99

Data for strains carrying pCXlif were obtained after induction with xylose (see Section 2). Values below 0.08 are not significant due to experimental setup (see also text).

Table 2
MIC values (μg ml^{-1}) of *S. aureus* strains to lysostaphin and methicillin after induction with xylose

Strain	Lysostaphin	Methicillin
BB270	0.125	2–3
BB270/pCXlif	2	2
BB270/pCX26Δlip	0.06	2–3
BB815	0.125	0.75
BB815/pCXlif	2	1
BB815/pCX26Δlip	0.06	1
UK17	16	0.5
UK17/pCXlif	16	0.25
UK17/pCX26Δlip	16	0.19
AS145	16	0.032
AS145/pCXlif	16	0.032

4. Discussion

Lif, the lysostaphin immunity factor, is known to incorporate serine into the peptidoglycan and thus to confer resistance to lysostaphin [6,7]. Here we showed that Lif incorporated serine into BB270/pCXlif and BB815/pCXlif and increased lysostaphin resistance as expected. Complementation of the different *femAB* mutants clearly showed that Lif functioned as a FemB analogue, and could not substitute for FemA. Further analyses are needed to determine if Lif incorporates Ser-Ser, Ser-Gly or Gly-Ser. The total serine plus glycine content was lower in BB815/pCXlif than in BB270/pCXlif, suggesting that Lif was not able to complement all triglycine side chains. Since *femAB* forms an operon, the balanced synthesis of FemA and FemB may be important and therefore complementation *in trans* from a plasmid is possibly less efficient than *in cis*. FemA and FemB together with the postulated FemX [12] that attaches the first glycine may have to interact with each other to determine the correct length of the side chain. A rather intriguing finding was that methicillin resistance seemed not to be increased in the *femB* mutant by Lif, and also even seemed to be slightly adversely

influenced by serine incorporation into the side chain in wild-type BB270. This raises the question whether alteration of the composition of the pentaglycine side chain affects the activity of PBP2′ and reduces methicillin resistance.

Acknowledgments

We thank G. Thumm and F. Götz for the plasmids. Part of this work was supported by Swiss National Science Foundation Grant 31-42182.94.

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