YkrB is the main peptide deformylase in *Bacillus subtilis*, a eubacterium containing two functional peptide deformylases

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Peptide deformylation is an essential process in eubacteria. The peptide deformylase Def has been suggested to be an attractive target for antibacterial drug discovery. Some eubacteria including medically important pathogens possess two *def*-like genes. Until now, the functionality of both genes has been tested only in *Staphylococcus aureus* with the result that one gene copy was functional. Here, expression of two functional *def*-like gene products in *Bacillus subtilis* is demonstrated. Besides the *def* gene, which is chromosomally located close to the formyltransferase gene *fmt* and which was overexpressed and biochemically tested previously, *B. subtilis* possesses a second *def*-like gene, called *ykrB*. The encoded protein is 32% identical to the *def* gene product. It was shown that either *def* or *ykrB* had to be present for growth of *B. subtilis* in rich medium (each was individually dispensable). Studies with a *def/ykrB* double deletion strain with xylose-inducible *ykrB* copy demonstrated that, besides *def*, the gene *ykrB* is a second cellular target of deformylase inhibitors such as the antibiotic actinonin. The gene products exhibited similar enzymic properties, exemplified by similar inhibition efficacy of actinonin in biochemical assays. Antibiotic susceptibility tests with different deletion strains and Northern analyses indicated that YkrB is probably the predominant deformylase in *B. subtilis*. It was shown that duplication of the deformylase function does not lead to an increased actinonin-resistance frequency in comparison to *B. subtilis* mutants carrying only one deformylase gene.

Keywords: underexpression mutants, antibiotic resistance, formyltransferase, actinonin, antibacterial target

INTRODUCTION

In eubacteria, nascent polypeptide chains carry *N*-formylmethionine as the first amino acid, and this is removed during protein maturation by the action of two enzymes: the peptide deformylase (Def) and the methionine aminopeptidase (Map) (Adams, 1968; Sherman *et al.*, 1985). The deformylation is a requirement for the activity of Map (Solbiati *et al.*, 1999) and probably therefore an essential process in eubacteria, as shown in *Escherichia coli* (Mazel *et al.*, 1994; Meinnel & Blanquet, 1994) and *Staphylococcus aureus* (Margolis *et*

al., 2000). Only when the *N*-formyltransferase (Fmt), catalysing the formylation of the initiator methionyl tRNA (tRNA^{fMet}), is inactivated does the *def* gene seem to be non-essential for bacterial survival and can be deleted (Mazel *et al.*, 1994).

Formylated proteins exist not only in eubacteria, but also in mitochondria and plastids (Kozak, 1983); various plant mitochondria and chloroplasts possess Def activity (Braun & Schmitz, 1993; Shanklin *et al.*, 1995) but it has not been detected in mammalian mitochondria (for a review see Giglione *et al.*, 2000).

The *def* gene was first isolated from *E. coli* (Meinnel & Blanquet, 1993; Mazel *et al.*, 1994). Subsequently, several other *def* genes from Gram-negative and Gram-positive bacteria have been characterized on the genetic and/or biochemical level (Meinnel & Blanquet, 1994;

Abbreviations: fMAS, *N*-formylmethionine-alanine-serine; IC₅₀, inhibitor concentration which inhibits 50% of enzyme activity; K_d, dissociation constant; K_m, Michaelis constant; MAS, methionine-alanine-serine; MLS, macrolide/lincosamide/streptogramin.

	Motif 1	Motif 2	Motif 3
YKRB_BACSU	GVGLAAPQ	EGCLS	QHEIDHLNG
DEF(inactive)_SAU	AAGLCAPQ	EGSIT	LHIIDQMNG
DEF_BACST	GIGLAAPQ	EGCLS	QHEIDHLNG
DEF_SAU	GVGLAAPQ	EGCLS	QHEIDHLNG
DEF_BACSU	GVGLAAPQ	EGCLS	QHEMDHLVG
DEF_ECOLI	GIGLAATQ	EGCLS	QHEMDHLVG

Fig. 1. Multiple sequence alignment of the predicted catalytic domains of peptide deformylases. Class II deformylases: YKRB_BACSU (YkrB from *B. subtilis*, SWISS-PROT accession no. Q45495); DEF (inactive)_SAU [inactive Def variant translated from the genomic sequence of *S. aureus* NCTC 8325 generated at the Oklahoma University (Oklahoma City, OK, USA)]; DEF_BACST (Def from *B. stearothermophilus*, SWISS-PROT accession no. O31410); DEF_SAU (Def from *S. aureus*, Genpept accession no. AAG02249). Class I deformylases: DEF_BACSU (Def from *B. subtilis*, SWISS-PROT accession no. P94462); DEF_ECOLI (Def from *E. coli*, SWISS-PROT accession no. P27251). Three conserved motifs defining the catalytic domain are shown. Bold amino acid residues deviate from the conserved motifs (Meinzel *et al.*, 1997). The positions of the motifs in protein YKRB_BACSU are: motif 1, G58–Q65; motif 2, E108–S112; motif 3, Q152–G160. Along the complete amino acid sequence, YKRB_BACSU exhibits identities of 67% to DEF_BACST, 61% to DEF_SAU, 32% to DEF_BACSU, 30% to DEF (inactive)_SAU and 27% to DEF_ECOLI.

Mazel *et al.*, 1997; Meinzel *et al.*, 1997; Belouski *et al.*, 1998; Evans *et al.*, 1998; Huntington *et al.*, 2000; Chen *et al.*, 2000). Def homologues have been identified in all eubacteria, but not in *Archaea*, *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. They can also be found in eukaryotic parasites such as *Plasmodium falciparum* and *Trypanosoma* spp. (Meinzel, 2000), in higher plants such as *Arabidopsis thaliana* (Genpept accession no. CAB87633), in *Drosophila melanogaster* (Genpept accession no. AAF54540), as well as in mice and humans (GenBank accession nos BE303602 and AW499510), although their exact function, especially in the fruit fly and in mammals, is unknown. In conclusion, the Def protein, which is essential for bacterial but probably not for mammalian survival, is broadly conserved among living organisms. Thus Def has been suggested to be an attractive target for antibacterial (and eventually antiparasitic) drug discovery (Gigliante *et al.*, 2000; Meinzel, 2000).

Indeed, a potent peptide deformylase inhibitor with antibacterial activity has recently been identified (Chen *et al.*, 2000). Actinonin is active against both Gram-positive and Gram-negative bacteria, indicating a high degree of similarity among eubacterial deformylases. Based on amino acid sequence similarity, peptide deformylases can be divided into two subfamilies (Gigliante *et al.*, 2000). One is represented by the *E. coli* enzyme and many deformylases from Gram-negative bacteria (class I) and the second by enzymes from *Bacillus stearothermophilus* and many Gram-positive bacteria (class II). Meinzel *et al.* (1997) have already reported that *B. stearothermophilus* and *E. coli* peptide deformylases share close enzymic properties in spite of little sequence homology.

Some eubacteria possess two *def* genes, especially Gram-positive representatives. Recently, it was shown that in *S. aureus* only one of the two genes is responsible for peptide deformylation (Margolis *et al.*, 2000). In *Bacillus subtilis*, a *def* class I gene was cloned previously and shown to encode a functional peptide deformylase (Mazel *et al.*, 1997; Leiting *et al.*, 1998; Durand *et al.*, 1999; Huntington *et al.*, 2000). On the other hand, *B. subtilis* contains an additional deformylase-like gene, called *ykrB*, whose product is highly similar to the class II deformylase from *B. stearothermophilus* (Fig. 1). We therefore investigated whether this gene is also functional in *B. subtilis*. Here, we present data that *B. subtilis* harbours two functional peptide deformylases, in contrast to all other eubacteria studied so far. This is the first time that the biochemical and physiological roles of two functional Def proteins derived from one organism are compared.

METHODS

Materials, bacterial strains, growth conditions and plasmids.

Actinonin, lysozyme, xylose and fluorescamine were purchased from Sigma Aldrich; N-formylmethionine-alanine-serine (fMAS) and methionine-alanine-serine (MAS) were obtained from Bachem; ciprofloxacin was from Bayer. All other chemicals were of the highest commercial grade.

E. coli XL-1 Blue was used for cloning, and *E. coli* M15 for IPTG-induced protein overexpression (see Table 1). The gene replacement experiments were performed in *B. subtilis* strain 168, which we call the wild-type strain in our studies. In this work, we generated several *B. subtilis* deletion strains (see Table 1).

All strains were grown in LB medium (1% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl) with the appropriate selection. For antibiotic selection, the concentrations of antibiotics were 50 µg ampicillin ml⁻¹, 5 µg chloramphenicol ml⁻¹, 20 µg neomycin ml⁻¹, 0.5 µg erythromycin ml⁻¹ + 12.5 µg lincomycin ml⁻¹ [for selecting macrolide/lincosamide/streptogramin (MLS) resistance] and 100 µg spectinomycin ml⁻¹. Cell densities (OD₆₀₀) were recorded with the UV-vis spectrophotometer DU-64 (Beckmann).

To overexpress proteins, the vector pQE60 (Qiagen) was used. The plasmids pJH101 (Ferrari *et al.*, 1983) and pDG1731xyl were used for gene replacement experiments. Plasmid pDG1731xyl is a derivative of pDG1731 (Guerout-Fleury *et al.*, 1996). A 1485 bp DNA fragment containing the xylose-regulator gene *xylR* and the xylose-inducible promoter of gene *xylA* P_{xyIA} from *Bacillus megaterium* was amplified from plasmid pX (Kim *et al.*, 1996) using the primers XYL1 (5'-AGAGGATCCCCATTTCCCCCTTTG-3') and XYL2 (5'-ATCAGATCTATCAACGTGATATAGGTTTGC-3'). The PCR product's terminal *Bam*HI and *Bgl*II restriction sites were used to introduce it into the *Bam*HI site of pDG1731, so that genes could be cloned into the one remaining *Bam*HI site downstream of P_{xyIA}. Plasmid pBEST501 was used to obtain the neomycin-resistance cassette by restriction digestion with the enzymes *Xba*I and *Not*I (Itaya *et al.*, 1989). An erythromycin-resistance cassette was obtained from pDG1731 (Guerout-Fleury *et al.*, 1996) by amplification using the primers ERM1 (5'-ATCTCTAGACCCGGGCTTGATCCA-TGGATTACGCG-3') with a 5'-terminal *Xba*I site and ERM2 (5'-ATCGCGGCCGCTTACTTATTAAATAATTTATAG-

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/description*	Source or reference
Strains		
<i>E. coli</i>		
XL-1 Blue	<i>recA1 endA1 gyrA96 thi hsdR17</i> ($r_k^- m_k^+$) <i>supE44 relA1 $\lambda^- lac^- F$</i> (<i>proAB laqI^q</i> Δ M15 Tn10- <i>tet</i>)	Stratagene
M15	<i>nal^s str^s rif^s lacI ara gal mtl F⁻ recA⁺ uvr⁺ lon⁺</i> pREP4(Km ^R <i>laqI^q</i>)	Qiagen
<i>B. subtilis</i>		
168	<i>trpC2</i>	Anagnostopoulos & Spizizen (1961)
MHD101	Δ <i>def::nm trpC2</i>	This work
MHD104	Δ <i>def::nm ΔthrC::xylR-P_{xylA}-def-spc trpC2</i>	This work
MHY101	Δ <i>ykrB::nm trpC2</i>	This work
MHY103	Δ <i>def::nm ΔykrB::erm ΔthrC::xylR-P_{xylA}-ykrB-spc trpC2</i>	This work
Plasmids		
pQE60	<i>E. coli</i> expression vector; resistance: ampicillin	Qiagen
pJH101	Vector for gene replacement in <i>B. subtilis</i> ; resistance: ampicillin, chloramphenicol, tetracycline	Ferrari <i>et al.</i> (1983)
pDG1731	Vector for ectopic integration at the <i>thrC</i> locus in <i>B. subtilis</i> ; resistance: ampicillin, MLS, spectinomycin	Guerout-Fleury <i>et al.</i> (1996)
pX	Vector containing <i>xylR-P_{xylA}</i> from <i>B. megaterium</i> ; resistance: chloramphenicol, ampicillin	Kim <i>et al.</i> (1996)
pDG1731xyl	Vector containing <i>xylR-P_{xylA}</i> from <i>B. megaterium</i> for ectopic integration at the <i>thrC</i> locus in <i>B. subtilis</i> ; resistance: ampicillin, MLS, spectinomycin	This work
pBEST501	Vector carrying a neomycin-resistance cassette; resistance: ampicillin, neomycin	Itaya <i>et al.</i> (1989)

* *nm*, the neomycin-resistance gene; *erm*, the erythromycin-resistance gene; and *spc*, the spectinomycin-resistance gene.

CTATTG-3') with a 5'-terminal *NotI* site, which could be digested with the respective restriction enzymes. A summary of the plasmids used in this study is given in Table 1.

***E. coli* and *B. subtilis* genetics, and PCR protocols.** DNA purification, restriction digestion, ligation and transformation of *E. coli* were performed according to standard protocols (Sambrook *et al.*, 1989). Genetic techniques with *B. subtilis*, including transformation procedures, were performed as described by Harwood & Cutting (1990).

All PCR primers had a calculated melting temperature of approximately 60 °C. For cloning purposes, *Pwo* DNA polymerase (Roche) was used, and for colony PCR procedures, a mixture of *Taq* DNA polymerase (Roche) and Thermo Sequenase (Amersham) was used. Flanking regions and genes for cloning into expression vectors were amplified from 100 ng *B. subtilis* chromosomal DNA at primer concentrations of 1 µM and dNTP concentrations of 250 µM. Twenty-five reaction cycles of 30 s at 94 °C/30 s at 52 °C/1–2 min at 72 °C were carried out followed by a final 5 min incubation at 72 °C. Complementary 5'- and 3'-flanking regions of PCR products were assembled by mixing 1 µl of each PCR reaction as template for the second PCR with outward primers and under the same conditions as described before. Diagnostic PCR on bacterial colonies was performed as follows. Cell material was suspended in 50 µl H₂O. A PCR reaction was performed in 50 µl with 5 µl of the suspended colony and under the same conditions described above.

Deletion experiments in *B. subtilis*. Deletions of the *def* and *ykrB* wild-type loci were generated as follows. The 600 bp

regions upstream and downstream of the genes of interest were PCR-amplified using the following oligonucleotide combinations: DEF1A (5'-ATCGGATCCGCTGTTAACGCAAGTCAGCG-3')/DEF1B (5'-TGCGGCCGCTAAATCTAGACTCCGCAGGATGTGTGACGAC-3') and YKR1A (5'-ATCGGATCCTAGCGTCTTTCACGTTAAATCC-3')/YKR1B (5'-TGCGGCCGCTAAATCTAGACTCGATGTTTTCCATAGTAATCAAG-3') for amplification of the upstream regions, including 11 5'-codons of *def* and seven 5'-codons of *ykrB*, respectively, as well as DEF2A (5'-AGTC-TAGATTTAGCGGCCGCAATCTAAAATAAGTAAAT-ACTATACAG-3')/DEF2B (5'-ATCGGATCCAATTTTTC-TACCATATACATAATGG-3') and YKR2A (5'-AGTCT-AGATTTAGCGGCCGCATATTGTGTTTCCTTTCA-AAGAACC-3')/YKR2B (5'-ATCGGATCCGTTCTCGA-GGAATCGGCAG-3') for amplification of the downstream regions, including seven 3'-codons of *def* and 17 3'-codons of *ykrB*, respectively. The primers were designed such that the ends of the PCR products facing the 5'- and 3'-regions of the genes carried a complementary tag sequence (5'-AGTCT-AGATTTAGCGGCCGCA-3'). This tag sequence was used to assemble the products in a second PCR reaction using the outward primers. The outward primers contained terminal *Bam*HI restriction sites allowing cloning of the fusion-PCR product into the *Bam*HI site of pJH101. The sequence cloned into pJH101 internally contained the tag sequence, which could be cut by *Xba*I and *Not*I. Using these restriction enzymes, a neomycin-resistance (*Nm^R*) cassette derived from pBEST501 and an erythromycin-resistance cassette (*Em^R*) derived from pDG1731 could be integrated, respectively. The resistance cassettes did not include any transcription term-

ination sequences and were cloned in the same transcriptional orientation as the deleted genes.

The resulting pJH101 derivatives were transformed into *B. subtilis* 168. The transformants were tested for neomycin or erythromycin resistance and chloramphenicol sensitivity in order to find clones where the deletion marker was introduced into the chromosome by a double homologous recombination event. The clones' correct genotypes were determined according to the sizes of the PCR products obtained from colony PCRs with appropriate primers flanking the regions of recombination. Correct recombinant clones were tested for their growth rate in LB medium.

Ectopic expression of the gene *ykrB* under the control of the xylose-inducible promoter P_{xyIA} was achieved by subcloning the PCR product containing the *B. subtilis* gene into the expression vector pDG1731xyl [PCR primer pair: YKRBR (5'-ATCGGATCCATGATTACTATGGAAAACATCGTAC-3')/YKRBT (5'-ATCGGATCCTTAGCGCTCAATTGCG-ATTGC-3')] and subsequent integration at the *thrC* locus of the *B. subtilis* *def*-deletion mutant by marker exchange as described elsewhere (Guerout-Fleury *et al.*, 1996). The chromosomal integration obtained with pDG1731xyl was marked with the spectinomycin-resistance (Spc^R) determinant. After ectopic integration of *ykrB* under the control of P_{xyIA} , the *B. subtilis* *def*-deletion mutant was transformed with a pJH101 derivative in order to delete the *ykrB* wild-type locus. This time the transformants were selected on selective media containing 0.25% (w/v) xylose. Clones resistant to spectinomycin (marker of xylose-inducible *ykrB* copy) and erythromycin (marker for the *ykrB* wild-type locus deletion), but sensitive to MLS and chloramphenicol (markers for the presence of pDG1731 and pJH101 sequences), were selected. The clones' correct genotypes were again verified using diagnostic colony PCRs with appropriate primers. A correct recombinant clone was tested for its ability to grow on LB medium without xylose.

Ectopic expression of the gene *def* under the control of the xylose-inducible promoter P_{xyIA} was achieved by subcloning the PCR product containing the gene into the expression vector pDG1731xyl [PCR primer pair: DEFR (5'-ATCGGATCCATGGCAGTAAAAAAGGTCGTAC-3')/DEFT (5'-ATCGGATCCTCATCCTTCCATATCCGCTAG-3')] and subsequent integration at the *thrC* locus of the *B. subtilis* *def*-deletion mutant by marker exchange as described before. The strain obtained served as control for detection of high expression of *def* in Northern analyses.

RNA preparation and Northern analyses. Total RNA was isolated from *B. subtilis* with the 'Qiagen RNeasy Mini kit'. Cells were grown in 10 ml LB medium at 37 °C until they reached OD_{600} 0.5. An equal volume of ice-cold killing buffer (5 mM $MgCl_2$; 20 mM NaN_3 ; 20 mM Tris/HCl, pH 7.5) was added. The cells were harvested and resuspended in 1 ml lysis buffer (25%, w/v, saccharose; 1 mg lysozyme ml^{-1} ; 250 μ M EDTA; 20 mM Tris/HCl, pH 8.0). After incubation on ice for 10 min and centrifugation at 4000 g at 4 °C for 5 min, the pellet was resuspended in 100 μ l TE buffer (1 mM EDTA; 10 mM Tris/HCl, pH 8.0). The following isolation steps were performed according to the manufacturer's instructions. The obtained RNA (100 μ g) was dissolved in H_2O and stored at -80 °C.

Digoxigenin-labelled RNA probes were produced by run-off transcription with 1 μ g PCR product as template using the 'DIG RNA labelling kit' with digoxigenin-labelled UTP according to the manufacturer's instructions (Roche). The PCR product representing gene *def* was generated with

primers DEFR (see above) and DEFTT7 (5'-CTAATACG-*ACTCACTATAGGGAGACATCCTTCCATATCCGCTAG-3'*); the product representing *ykrB* was obtained with primers YKRBR (see above) and YKRBT7 (5'-CTAATACG-*ACTCACTATAGGGAGAGCGCTCAATTGCGATTGC-3'*). The 5'-ends of the 3'-terminal primers contained the promoter of the T7 RNA polymerase (see letters in italics within the primer sequences mentioned before).

Total RNA (10 μ g) was electrophoresed through formaldehyde gels (Sambrook *et al.*, 1989). The RNA size standard was obtained from Gibco-BRL. After electrophoresis, RNA was transferred to a GeneScreen Plus nylon membrane (NEN) by capillary transfer using 20 $\times$ SSC (3 M NaCl, 0.3 M sodium citrate). RNA was then stained with 0.1% methylene blue. Hybridization and subsequent detection of the RNA probe with CDP-Star as substrate were performed according to 'The Dig System User's Guide for Filter Hybridization' (Roche). Briefly, the overnight hybridization with 300 ng labelled probe ml^{-1} as well as the four 15 min washing steps (twice in 2 $\times$ SSC, 0.1% SDS and twice in 0.1 $\times$ SSC, 0.1% SDS) were carried out at 60 °C. The chemiluminescence signals on the membrane were measured in the Lumi Imager F1 (Roche).

Antibiotic susceptibility tests. Microdilution MICs were determined against *B. subtilis* strains in 96-well microtitre plates in LB medium containing serial dilutions (twofold) of antibiotics. A starting inoculum of $0.5-1.0 \times 10^5$ c.f.u. ml^{-1} derived from exponentially growing cells was used. The MIC was the lowest concentration of drug that yielded no visible growth after incubation for 18-24 h at 37 °C. End points were determined by measuring the OD_{600} with the microtitre plate reader EL312e (Bio-Tec Instruments).

Growth curves of *B. subtilis* strains with actinonin treatment were obtained as follows. An overnight culture of *B. subtilis* was diluted 100-fold into fresh LB medium and grown to OD_{600} 0.1. The cell cultures were diluted by 10-fold into 50 ml fresh LB medium and incubated at 37 °C. At OD_{600} 0.4 (10^8 c.f.u. ml^{-1}), actinonin was added to the culture (final concentration 20 μ g ml^{-1}) and growth of the *B. subtilis* strains was continuously monitored spectrophotometrically.

Isolation of actinonin-resistant mutants. Spontaneous actinonin-resistant mutants were isolated by plating approximately 10^8 c.f.u. from exponentially growing cells of *B. subtilis* on LB agar plates containing 32 μ g actinonin ml^{-1} . The plates were incubated at 37 °C overnight. Colonies that grew were transferred to 5 ml actinonin-free LB medium and grown overnight before again determining the MICs of actinonin for such clones. Using this isolation procedure, the frequency of actinonin-resistant clones was determined.

Cloning, expression and purification of Def and YkrB. The *B. subtilis* genes *def* and *ykrB* were PCR-amplified from genomic DNA of *B. subtilis* 168 and cloned into expression vector pQE60 (Qiagen) using *NcoI* and *BamHI* restriction sites. The oligonucleotide combinations were as follows: DEF1 (5'-GCGCCCATGGCAGTAAAAAAGGTCGTAC-3')/DEF2 (5'-GCGCAGATCTTCCCTTCCATATCCGCTAG-3') and YKR1 (5'-GCGCCCATGGTCTACTATGGAAAACATCGTAC-3')/YKR2 (5'-GCGCAGATCTGCGCTCAATTGCGATTGCATT-3'). The resulting plasmid constructs were confirmed by DNA sequence analysis and used to transform *E. coli* M15. The M15 strains containing the expression vectors were grown exponentially up to OD_{600} 0.5 at 37 °C in LB-ampicillin-kanamycin medium and then induced with 1 mM IPTG for 4 h before harvesting by centrifugation. The proteins were purified in a single step and under native conditions using nickel-nitrilotriacetic acid columns according to the manu-

facturer's instructions (Qiagen; QIAexpressionist manual). Subsequently, nickel sulfate was added to a final concentration of 10 mM. Proteins were quantified using BSA as standard (Bradford, 1976). The purified proteins, which were >95% pure as estimated by SDS-PAGE, were directly tested in enzymic assays.

Enzyme assay. The deformylase activity was measured in 384-well microtitre plates. Twenty microlitres of buffer (50 mM HEPES, pH 7.0; 10 mM NaCl; 0.1% Triton X-100) was mixed with 20 µl enzyme solution (end concentration in 60 µl assay volume: 450 nM) and incubated for 20 min at room temperature (RT). The reaction was started by addition of appropriate amounts of substrate (fMAS) and incubated for 60 min at RT. The free amino-terminus of the deformylated product MAS was measured by addition of 20 µl fluorescamine solution (2.5 µg ml⁻¹ 100% DMSO). Fluorescence was measured using Spectrafluor Plus (Tecan) with the excitation wavelength at 390 nm and the emission wavelength at 465 nm. To determine product concentrations, standard curves were established by measuring defined concentrations of MAS mixed with fluorescamine solution spectrophotometrically.

RESULTS AND DISCUSSION

B. subtilis contains two *def*-like genes and one of these has to be present for growth to occur

B. subtilis possesses two *def*-like genes, *def* and *ykrB*. We successfully deleted each gene by replacement with neomycin cassettes engineered to enable non-polar insertions. The *def*-deletion mutant MHD101 as well as the *ykrB*-deletion mutant MHY101 exhibited growth rates in LB medium identical to that of wild-type *B. subtilis* (Fig. 2). Obviously, both genes were each individually non-essential for growth. Subsequently, we

Fig. 2. Growth of *B. subtilis* mutants in LB medium. Growth of the following strains was monitored: *B. subtilis* wild-type (wt), the *def*-deletion strain MHD101, the *ykrB*-deletion strain MHY101, and the *defykrB* double deletion strain MHY103 with xylose-inducible *ykrB* copy. The wild-type strain and the mutant MHY103 were also grown in the presence of xylose. The experiment was performed as follows. A volume of 5 ml xylose-free LB medium was inoculated with fresh colonies, grown on LB-agar plates with 0.25% xylose, and incubated overnight at 37 °C. Flasks containing 50 ml LB with and without 0.25% (w/v) xylose were inoculated with diluted aliquots of the overnight culture (start OD₆₀₀ 0.01) and incubated at 37 °C. Growth was monitored by optical density measurements. ●, MHY103 (LB); ■, MHY103 (LB + xylose); ▽, MHD101 (LB); ◇, MHY101 (LB); △, wt (LB); □, wt (LB + xylose).

tried to generate a mutant carrying deletions in *def* as well as in *ykrB*. We tried to replace the respective second *def*-like gene with an erythromycin-resistance cassette in both deletion strains MHD101 and MHY101. We were not able to generate such double deletions.

After integration of *ykrB* downstream from a xylose-inducible promoter into the *thrC* region of the *def*-deletion strain MHD101, we were able to delete the *ykrB* wild-type locus. The generated mutant MHY103, which carried deletions in the natural loci of *def* and *ykrB* and harboured the xylose-inducible *ykrB* copy, grew with xylose such as the *B. subtilis* wild-type, but exhibited a growth arrest over 5 h in xylose-free LB medium under the conditions described in Fig. 2. Afterwards, we could recover growth of strain MHY103 and harvest cells for RNA preparation (OD₆₀₀ 0.5 reached after 10 h), because the xylose-promoter system is leaky (Rygus & Hillen, 1992). We demonstrated that *ykrB* transcripts could not be detected in Northern analyses using digoxigenin-labelled probes (see Methods). In contrast, in the presence of 0.25% xylose a high amount of *ykrB* transcripts was found in strain MHY103 (Fig. 3). We can conclude that the function encoded by *def* and *ykrB*, which complement each other, is essential for survival of *B. subtilis*.

Both proteins Def and YkrB catalyse peptide deformylation

The genes *def* and *ykrB* were PCR-amplified from genomic DNA of *B. subtilis* and cloned into the expression vector pQE60. They were overexpressed in *E. coli* M15 and purified as proteins with C-terminal His₆-tags. The proteins were tested for their peptide deformylase activity (see Methods). In order to keep the activity of the proteins stable, we saturated the enzymes with NiSO₄ (Giglione *et al.*, 2000). This treatment did not significantly change the enzymic properties of the originally purified proteins as we have tested this previously (data not shown). We could show that both proteins (Def and YkrB) catalyse the deformylation of fMAS, the substrate which we used in our enzymic deformylase assay (Fig. 4). The enzymes Def and YkrB exhibited similar apparent Michaelis constants (*K_m*) for fMAS of 2.3 mM and 3.0 mM, respectively. Under conditions fully saturated with substrate, YkrB was 2.5 times faster than Def (apparent turnover number = 0.17 s⁻¹ and 0.07 s⁻¹, respectively). Besides similar *K_m* values, the similarity in enzymic properties could also be demonstrated by the inhibition of their activities in the presence of actinonin. Under the same assay conditions, the actinonin concentrations which could inhibit 50% of enzyme activity (IC₅₀) were 95 nM in the case of Def and 130 nM in the case of YkrB (Fig. 4).

Def and YkrB possess similar enzymic properties in cellular as well as cell-free systems

Chen *et al.* (2000) have already demonstrated that the expression level of a deformylase in *E. coli* can be correlated with the antibacterial activity of actinonin,

Fig. 3. (a) Gene structure of the chromosomal *thrC* region with integrated xylose-inducible copy of *ykrB* such as in *B. subtilis* strain MHY103. Genes are represented by bold arrows showing the direction of transcription. Gene names are given directly below the gene map. An internal fragment of *thrC* is replaced by the xylose regulator gene *xylR*, the gene *ykrB* located downstream from the xylose-inducible promoter P_{xylA} , and the spectinomycin-resistance gene *spc*. There is no strong terminator located downstream of *ykrB*. After xylose induction, *ykrB*-containing transcripts with different lengths can be detected in Northern analyses [see (b), lane 5]. The detected transcript lengths are represented by thin arrows (marked with an asterisk) and correlated with the gene map. The base pair positions in the *thrC* region are given underneath. (b) Northern hybridization of RNA isolated from the following *B. subtilis* strains grown in LB medium: wild-type strain (lanes 1 and 6); *def*-deletion strain MHD101 (lanes 2 and 7); *ykrB*-deletion strain MHY101 (lanes 3 and 8); *defykrB* double deletion strain MHY103 with ectopic, xylose-inducible copy of *ykrB* grown in the absence (lane 4) and presence (0.25%, w/v; lane 5) of xylose; *def*-deletion strain MHD104 with ectopic, xylose-inducible copy of *def* (see Table 1) grown in the absence (lane 9) and presence (0.25%, w/v; lane 10) of xylose. A digoxigenin-labelled RNA complementary to *ykrB* was used as hybridization probe from lanes 1 to 5, and a digoxigenin-labelled RNA complementary to *def* was used as hybridization probe from lanes 6 to 10. Lengths of the RNA size standard are given on the left side; transcript lengths detected with the hybridization probes are shown on the right side. While a *ykrB* transcript could be detected [length approx. 0.7 kb, lanes 1 and 2], no clear hybridization signal could be obtained using the *def*-antisense probe. Ectopic overexpression of *ykrB* and *def* after xylose induction could be monitored (lanes 5 and 10). The *def*- and *ykrB*-containing transcripts have comparable sizes, because the *def* gene is only 70 nt shorter than *ykrB*. The transcripts probably contain only *ykrB* or *def* (approx. 0.6 and 0.8 kb); *ykrB* or *def* and parts of *spc* (approx. 1.1 and 1.4 kb); *ykrB* or *def* and complete *spc* (approx. 1.7 kb); *ykrB* or *def*, *spc* and *thrC*-3'-end (approx. 2.2 kb); *ykrB* or *def*, *spc*, *thrC*-3'-end and *thrB* [approx. 3.4 kb; see also part (a)].

Fig. 4. Enzymic measurements with C-terminal His₆-tagged YkrB and Def saturated with Ni. (a) Enzymic activity plotted with increasing concentrations of substrate fMAS. The curves are the best fit of the data to the Michaelis-Menten equation. The apparent K_m values are 2.3 mM in the case of Def and 3.0 mM in the case of YkrB. The apparent V_{max} values are $2.0 \mu\text{M min}^{-1}$ in the case of Def (enzyme concentration = $0.45 \mu\text{M}$; deduced turnover number = 0.07 s^{-1}) and $4.6 \mu\text{M min}^{-1}$ in the case of YkrB (enzyme concentration = $0.45 \mu\text{M}$; deduced turnover number = 0.17 s^{-1}). Error bars represent the standard deviation of data reproduced four times. (b) Dose-response relationship of actinin inhibition against Def and YkrB. Deformylation activities of 450 nM enzymes were measured in the presence of 1.22 mM fMAS and increasing concentrations of actinin. The deduced IC_{50} values for Def and YkrB are given in the figure. Error bars represent the standard deviation of triplicate data.

Fig. 5. Antibiotic susceptibility tests. (a) Microdilution MICs of actinonin in LB medium with different concentrations of xylose for the *B. subtilis* wild-type strain and the *def/ykrB* double deletion strain MHY103 with xylose-inducible *ykrB* copy (see Methods). At low xylose concentrations, the actinonin MIC for strain MHY103 was 0.003–0.012 $\mu\text{g ml}^{-1}$, while the MIC of 0.9 $\mu\text{g ml}^{-1}$ at high xylose concentrations (> 0.25%) was only one dilution step (1:2) lower than the MIC for the wild-type strain (1.9 $\mu\text{g ml}^{-1}$). For comparison, the MIC values of ciprofloxacin were determined. Bars indicate the minimal and maximal MIC value determined for a given condition if there were differences among three experiments performed. ■, Wild-type + actinonin; ▼, MHY103 + actinonin; ○, wild-type + ciprofloxacin; ◇, MHY103 + ciprofloxacin. (b) Growth curves of *B. subtilis* strains in LB medium with and without actinonin. After addition of 20 $\mu\text{g actinonin ml}^{-1}$ at OD₆₀₀ 0.4 (see Methods), growth of the following strains was monitored: *B. subtilis* wild-type strain, the *def*-deletion strain MHD101 and the *ykrB*-deletion strain MHY101. For comparison, growth of the strains without actinonin is shown (represented by one single curve). The mean OD₆₀₀ values are given with bars representing the minimum and maximum OD₆₀₀ obtained in three independent experiments. The microdilution MICs of actinonin in LB medium are 1.9 $\mu\text{g ml}^{-1}$ for *B. subtilis* wild-type and strain MHD101, and 0.3–0.5 $\mu\text{g ml}^{-1}$ for strain MHY101. ▲, Strains – actinonin; ▼, wild-type + actinonin; ○, MHD101 + actinonin; □, MHY101 + actinonin.

the most potent deformylase inhibitor known so far. We repeated this experiment in *B. subtilis* with the *def/ykrB* double deletion mutant MHY103, in order to confirm that YkrB is also a deformylase targeted by actinonin inside the cell. We determined the dependence of the MIC values for the mutant on different xylose concentrations, the inducer for *ykrB* expression. We obtained

no correlation between the MIC values of the control compound ciprofloxacin, which does not inhibit deformylase activity, and the xylose concentration. However, a strong correlation was observed between MIC values of actinonin and the xylose concentration, showing that YkrB is indeed targeted by actinonin in the cellular system (Fig. 5a).

At high xylose concentrations (> 0.25%) no further increase in MIC values could be detected (Fig. 5a). Similar results were reported by Chen *et al.* (2000), who used an *E. coli* strain with an arabinose-inducible *def* gene. This plateau in MIC values might indicate that at high inducer concentrations the deformylase activity of the induced protein is no longer proportional to the inducer concentration.

The *B. subtilis* peptide deformylase Def has already been successfully isolated and tested for deformylase function (Leiting *et al.*, 1998; Durand *et al.*, 1999; Huntington *et al.*, 2000). Peptide thiol-like deformylase inhibitors exhibiting a wide range of dissociation constants (K_i) against Def of *B. subtilis* possessed MIC values for *B. subtilis* which could be correlated with the K_i values of the inhibitors (Huntington *et al.*, 2000). This is only possible if YkrB is inhibited as efficiently as Def by peptide thiols, since our data demonstrate that *def* alone is not essential for growth of *B. subtilis* and *ykrB* also encodes a functional deformylase. Obviously, the class I and class II deformylases of *B. subtilis* possess similar enzymic properties in a cellular as well as in a cell-free system.

B. subtilis probably contains more YkrB than Def

When we tested the sensitivity of *B. subtilis* carrying deletions in either *def* or *ykrB*, we found that the *ykrB*-deletion mutant MHY101 was more sensitive to actinonin than the *def*-deletion strain MHD101. While the MIC values for MHY101 were 0.3–0.5 $\mu\text{g ml}^{-1}$, the MIC values of MHD101 correspond to the MIC values for the wild-type strain (1.9 $\mu\text{g ml}^{-1}$). When we compared the growth curves of mutants and wild-type strain with actinonin treatment, an increased sensitivity of the *ykrB*-deletion mutant MHY101 in comparison to the *def*-deletion strain MHD101 could also be demonstrated (compare strains MHY101 + actinonin and MHD101 + actinonin in Fig. 5b). Since Def and YkrB possess similar enzymic properties and are inhibited by actinonin with the same efficiency, the simplest and most probable explanation for the different sensitivity of deletion mutants to actinonin is that *B. subtilis* contains more YkrB proteins than Def proteins. Thus deletion of *ykrB* makes *B. subtilis* more sensitive to actinonin than deletion of *def*.

Northern analyses support the idea that *ykrB* is more highly expressed in *B. subtilis* than *def* (Fig. 3). We were able to detect a monocistronic transcript in *B. subtilis* wild-type and in the *def*-deletion strain MHD101, which disappeared in the *ykrB*-deletion strain MHY101. In contrast, we were not able to clearly identify *def*

transcripts in *B. subtilis* wild-type and in the *ykrB*-deletion mutant using digoxigenin-labelled probes, although *def*-containing transcripts in a strain over-expressing *def* could be detected using the same probe.

Taking the results of the actinonin sensitivity tests and the Northern analyses together, we can conclude that YkrB is probably the predominant deformylase in *B. subtilis*, although final experiments on the protein level have to be performed to strengthen this conclusion.

Actinonin resistance of organisms with two deformylases

Besides *B. subtilis*, there are other organisms including important pathogens such as *S. aureus* which contain two deformylase genes. In *S. aureus*, one of the two *def* copies (the one which is associated with *fnt* in the chromosome) exhibits deviations in the catalytically important sequence motifs (Fig. 1) and has been identified to be inactive (Margolis *et al.*, 2000). In *B. subtilis*, both Def-like proteins harbour conserved sequence motifs (Fig. 1) and are indeed functional. Several other pathogens, such as *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*, could also possess two functional deformylases (Margolis *et al.*, 2000; Giglione *et al.*, 2000). Redundancy of essential functions in the bacterial cell can have serious implications for generation of resistance to drugs targeting the respective enzymes. Resistance can simply be achieved through a gene dosage effect or by mutations in which one copy of the gene encodes an enzyme resistant to the antibiotic while the other one continues to function normally. We therefore compared the actinonin-resistance frequency of *B. subtilis* strains carrying only one *def*-like gene (MHD101 and MHY101) to that of *B. subtilis* 168. Remarkably, actinonin resistance arose in each strain at approximately the same frequency of 10^{-5} . (The frequencies for each strain were determined twice.) One reason for this phenomenon could be that alterations in the *fnt* genes are the favoured resistance mechanisms even in organisms with two functional deformylases. The lack of formylation makes deformylation dispensable for the cell. This mode of resistance has been described in *S. aureus*, which harbours only one functional deformylase (Margolis *et al.*, 2000). Sequence analyses of the relevant chromosomal loci in the resistant *B. subtilis* strains will be necessary to confirm the hypothesis.

Conclusions

From the evolutionary point of view, two classes of deformylases evolved which possess distant sequence similarity to each other, but which retain similarity in the essential sequence motifs (Fig. 1) and in 3-D structure (Dardel *et al.*, 1998). Comparison of the two deformylases from *B. subtilis* illustrates that they possess remarkably similar enzymic properties and can be targeted by the antibiotic actinonin with the same efficiency. Actinonin indeed harbours antibacterial activity against a broad spectrum of bacteria which contain

either class I or class II deformylases. *B. subtilis* is the first organism where expression and functionality of both *def*-like genes has been demonstrated. Although each of the two genes individually retains viability of *B. subtilis*, the gene product YkrB probably represents the predominant deformylase species in the organism. The presence of two deformylases in the cell does not necessarily increase the resistance frequency to antibiotics targeting peptide deformylation, such as actinonin. As in other organisms, resistance mechanisms not directly connected to the target also seem to be favoured in *B. subtilis*.

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