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<sub>fMet</sub>), 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;...
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.** Actinomycin, 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/lincomamide/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., 1996) 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 from Bacillus megaterium was amplified from plasmid pX (Kim et al., 1996) using the primers XYL1 (5'-AGAGGATCCATTTCCATTTTCTG-3') and XYL2 (5'-ATCGATCTATACGGATATAGTTTG-3'). The PCR product’s terminal BamHI and BglII restriction sites were used to introduce it into the BamHI site of pDG1731, so that genes could be cloned into the one remaining BamHI site downstream of PₓylA. Plasmid pBEST501 was used to obtain the neomycin-resistance cassette by restriction digestion with the enzymes XbaI and NotI (Itaya et al., 1989). An erythromycin-resistance cassette was obtained from pDG1731 (Guerout-Fleury et al., 1996) by amplification using the primers ERM1 (5'-ATCTCTAGACCCGGCTTGATCCCATGATTACGG-3') with a 5'-terminal XbaI site and ERM2 (5'-ATCGCGCCGCTTACTTATTTATTATAGG-3') with a 5'-terminal NotI site.
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.

**Table 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype/description*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>recA1 endA1 gyrA96 thi bsdR17 (rE· mC) supE44 relA1 × lac - F (proAB lacZΔM15 Tn10-tet)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>M15</td>
<td>ndr str rif lac ara gal mtl F- recA ura lon pREP4(Km l laq l)</td>
<td>Qiagen</td>
</tr>
<tr>
<td>B. subtilis 168</td>
<td>trpC2</td>
<td>Anagnostopoulos &amp; Spizizen (1961)</td>
</tr>
<tr>
<td>MHD101</td>
<td>Δdef::nm trpC2</td>
<td>This work</td>
</tr>
<tr>
<td>MHD104</td>
<td>Δdef::nm ΔthrC::xylR-P-xylA-def-spc trpC2</td>
<td>This work</td>
</tr>
<tr>
<td>MHY101</td>
<td>ΔykrB::nm trpC2</td>
<td>This work</td>
</tr>
<tr>
<td>MHY103</td>
<td>Δdef::nm ΔykrB::erm ΔthrC::xylR-P-xylA-ykrB-spc trpC2</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pQE60</td>
<td>E. coli expression vector; resistance: ampicillin</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pJH101</td>
<td>Vector for gene replacement in <em>B. subtilis</em>; resistance: ampicillin, chloramphenicol, tetracycline</td>
<td>Ferrari et al. (1983)</td>
</tr>
<tr>
<td>pDG1731</td>
<td>Vector for ectopic integration at the <em>thrC</em> locus in <em>B. subtilis</em>; resistance: ampicillin, MLS, spectinomycin</td>
<td>Guerout-Fleury et al. (1996)</td>
</tr>
<tr>
<td>pX</td>
<td>Vector containing xylR-P-xylA from <em>B. megaterium</em>; resistance: chloramphenicol, ampicillin</td>
<td>Kim et al. (1996)</td>
</tr>
<tr>
<td>pDG1731xyl</td>
<td>Vector containing xylR-P-xylA from <em>B. megaterium</em> for ectopic integration at the <em>thrC</em> locus in <em>B. subtilis</em>; resistance: ampicillin, MLS, spectinomycin</td>
<td>This work</td>
</tr>
<tr>
<td>pBEST501</td>
<td>Vector carrying a neomycin-resistance cassette; resistance: ampicillin, neomycin</td>
<td>Itaya et al. (1989)</td>
</tr>
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*nm, the neomycin-resistance gene; erm, the erythromycin-resistance gene; and spc, the spectinomycin-resistance gene.*
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<sub>xyl</sub> was achieved by subcloning the PCR product containing the B. subtilis gene into the expression vector pDG1731xyl [PCR primer pair: YKBR (5′-ATCGGATCCATGATTACATATGGAAAAACATCGAC-3′)/YKRB (5′-ATCGGATCCATGACGCTAATTTGCGCGGCGATTGC-3′)] and subsequent integration at the tbrC locus of the B. subtilis def-deletion mutant by marker exchange as described elsewhere (Guerrout-Fleury et al., 1996). The chromosomal integration obtained with pDG1731xyl was marked with the spectinomycin-resistance (Sp<sup>ρ</sup>) determinant.

After ectopic integration of ykrB under the control of P<sub>xyl</sub>, 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<sub>xyl</sub> was achieved by subcloning the PCR product containing the gene into the expression vector pDG1731xyl [PCR primer pair: DEFIR (5′-ATCGGATCCATGATTACATATGGAAAAACATCGAC-3′)/DEFIR (5′-ATCGGATCCATGACGCTAATTTGCGCGGCGATTGC-3′)] and subsequent integration at the tbrC 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<sub>600</sub> 0.5. An equal volume of ice-cold killing buffer (5 mM MgCl<sub>2</sub>, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM Tris/HCl, pH 7.5) was added. The cells were harvested and resuspended in 1 ml lysis buffer (25%, w/v, sucrose; 1 mg lysozyme ml<sup>−1</sup>, 250 μM EDTA; 20 mM Tris/HCl, pH 8.0) for 10 min and centrifugation at 4000 g at 4 °C for 5 min, the pellet was resuspended in 100 μl 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 μg) was dissolved in H<sub>2</sub>O and stored at −80 °C.

Digoxigenin-labelled RNA probes were produced by run-off transcription with 1 μg 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 DEFTT (5′-CTATAGGACTCTACATTGGAGACATCCCCTTATTAGCCTAG-3′); the product representing ykrB was obtained with primers YKBR (see above) and YKRB (5′-CTTAATCAGCTCATAATAGGGAGACATCCCCTTATTAGCCTAG-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 μg) 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% 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<sup>−1</sup> as well as the four 15 min washing steps (twice in 2 x SSC, 0.1% SDS and twice in 0.1 x 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 x 10<sup>8</sup> c.f.u. ml<sup>−1</sup> 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<sub>600</sub> 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<sub>600</sub> 0.1. The cell cultures were diluted by 10-fold into 50 ml fresh LB medium and incubated at 37 °C. At OD<sub>600</sub> 0.4 (10<sup>8</sup> c.f.u. ml<sup>−1</sup>, actinonin was added to the culture (final concentration 20 μg ml<sup>−1</sup>) 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<sup>8</sup> c.f.u. from exponentially growing cells of B. subtilis on LB agar plates containing 32 μg actinonin ml<sup>−1</sup>. 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′-GGCCTTGCATGAATGAAAAACGTCGAC-3′)/DEF2 (5′-GGCAGATCCTCTCTCTCATCCGTAG-3′) and YKR1 (5′-GGCCCTATGTTACTATATGGAAAAACATCGAC-3′)/YKR2 (5′-GGCGAGATCCTGTGCTAATTTGCGCGGCGATTGC-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<sub>600</sub> 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 deformed product MAS was measured by addition of 20 µl fluorescamine solution (2·5 µg ml⁻¹ 100% DMSO). Fluorescence was measured using Spectramax 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 MHY101 as well as the *ykrB*-deletion mutant MHY103 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 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 (*Kₘ*) 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 *Kₘ* values, the similarity in enzymic properties could also be demonstrated by the inhibition of their activities in the presence of actinomycin. Under the same assay conditions, the actinomycin 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 actinomycin,
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 xyIR, 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 His$_6$-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 $\mu$M min$^{-1}$ in the case of Def (enzyme concentration = 0.45 $\mu$M; deduced turnover number = 0.07 s$^{-1}$) and 4.6 $\mu$M min$^{-1}$ in the case of YkrB (enzyme concentration = 0.45 $\mu$M; deduced turnover number = 0.17 s$^{-1}$). Error bars represent the standard deviation of data reproduced four times. (b) Dose–response relationship of actinonin 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 actinonin. The deduced $IC_{50}$ values for Def and YkrB are given in the figure. Error bars represent the standard deviation of triplicate data.
Strain MHY103 was 0.003–0.012 µg ml⁻¹, while the MIC of 0.9 µg 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 µg 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.

**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 *defykrB* 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 µg ml⁻¹, while the MIC of 0.9 µg 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 µg 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 µg 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 µg ml⁻¹ for *B. subtilis* wild-type and strain MHD101, and 0.3–0.5 µg ml⁻¹ for strain MHY101. **Strains – actinonin; ▼, wild-type + actinonin; ○, MHD101 + actinonin; □, MHY101 + actinonin.**

There was 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 inductor concentrations the deformylase activity of the induced protein is no longer proportional to the inductor 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 (Kᵣ) against Def of *B. subtilis* possessed MIC values for *B. subtilis* which could be correlated with the Kᵣ 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 µg ml⁻¹, the MIC values of MHD101 correspond to the MIC values for the wild-type strain (1.9 µg 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 fmt 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 fmt 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 harbourres 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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**REFERENCES**


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