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Note

Carbohydrate esterase family 4 enzymes: substrate specificity

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Abstract

The substrate specificity of selected enzymes classified under Carbohydrate Esterase family 4 (CE4) has been examined. Chitin deacetylase from *Mucor rouxii* and both a native and a truncated form of acetyl xylan esterase from *Streptomyces lividans* were found to be active on both xylan and several soluble chitinous substrates. Furthermore, the activities of all enzymes examined were significantly increased in the presence of Co^{2+} when chitinous substrates were employed. However, the presence of this metal ion did not result in enhancing the activities of the enzymes when xylan was used as substrate. An acetyl xylan esterase from *Bacillus pumilus*, classified under Carbohydrate Esterase family 7, was found to be inactive towards all chitinous substrates tested. Finally, all enzymes examined were inactive towards cell wall peptidoglycan.

Keywords: Chitin deacetylase; Chitin; Acetyl xylan esterase; Xylan; Peptidoglycan

Carbohydrate Esterase family 4, according to Henrissat classification[1,2] includes chitin deacetylases (EC 3.5.1.41), rhizobial NodB chitooligosaccharide deacetylases (EC 3.5.1.-), peptidoglycan *N*-acetylglucosamine deacetylases (EC 3.1.1.-), acetyl xylan esterases (EC 3.1.1.72) and xylanases A, C, D, E (EC 3.2.1.8). All five members of this family catalyse the hydrolysis of either N-linked acetyl groups from N-acetylglucosamine residues (chitin deacetylases, rhizobial NodB chitooligosaccharide deacetylases and peptidoglycan *N*-acetylglucosamine deacetylases), or O-linked acetyl groups from O-acetylxylose residues (acetyl xylan esterases and xylanases A, C, D, E) of their substrates, namely, chitin, NodB factors, peptidoglycan and acetyl xylan, respectively. Although several genes of Carbohydrate Esterase family 4 enzymes have been cloned, characterized and expressed, the native structure of any of these enzymes has not been presently elucidated. All enzymes share a universal conserved region, which has been assigned as the nodB homology domain, due to its similarity to NodB proteins[3] (Fig. 2). It has been reported that the NodB domain present in *Cellulomonas fimi* xylanase D was functional, deacetylating xylan substrates and thus contributing to the efficient hydrolysis of acetylated xylan by xylanases.[4] In this study, we have performed a more systematic study on the substrate specificity of Carbohydrate Esterase family 4 enzymes, considering the sequence similarities of the enzymes and the structural similarities of their respective substrates.

Selected members of Carbohydrate Esterase family 4 namely chitin deacetylase (CDA) from *Mucor rouxii*, acetyl xylan esterase from *Streptomyces lividans* and a truncated form of this enzyme lacking the xylan binding domain were examined in order to test their effectiveness to deacetylate substrates related to this enzyme family namely glycol chitin, chitin 50 (a 40% deacetylated chitin with an average M_r of approx 160,000) and *N*-acetylchitotetraose as well as xylan and peptidoglycan (Fig. 1).

Chitin deacetylase from *M. rouxii* was effective in deacetylating xylan and all soluble chitin substrates

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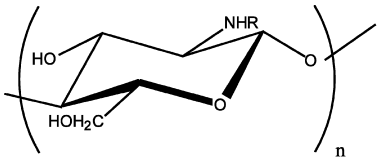
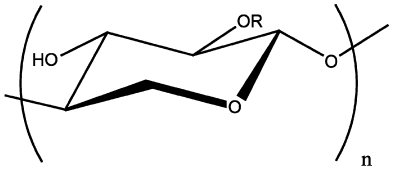
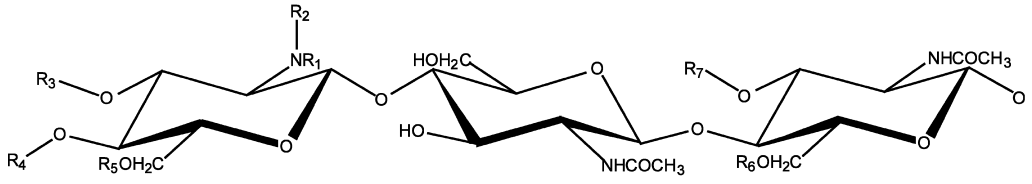


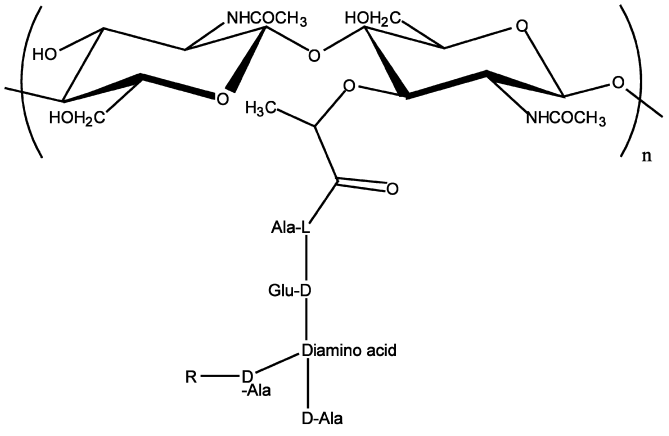
Chitin R = $COCH_3$ **Chitosan** R = H



Acetyl xylan R = $OCOCH_3$ or R = H



Nod factor



Peptidoglycan

Fig. 1. Structures of Chitin, Chitosan, Acetyl xylan, Peptidoglycan and Nod factor. Nod factor R_1–R_7, $n = 1$–3: the type of substituent groups reported have been found to vary depending to the rhizobial strain. Nod factors produced by all rhizobia have the same structure: generally, four or five N-acetyl glucosamines residues, β(1,4) linked with the terminal non-reducing sugar *N*-acetylated with a fatty acid generally of 16 or 18 carbon atoms. Different rhizobial species produce characteristic Nod factor structures with chemical substitutions on the reducing and non-reducing sugars, and variations in the structure of the acyl chain. The range and nature of the Nod factor structures and the amount produced by a rhizobial strain are important for nodulation and host range specificity.[19,20] Peptidoglycan R: may be connected to the peptide interbridge or to the diaminopimelic acid in another tetrapeptide.

examined. (Table 1). Similarly acetyl xylan esterase from *S. lividans* was also effective in deacetylating all chitin substrates examined in addition to xylan (Table 1). The truncated form of acetyl xylan esterase was also effective in deacetylating all substrates examined, indicating that the presence of the xylan binding domain similarly to other cases, e.g., chitin binding domains of chitinases, is not required for hydrolysis of the substrates.[5] Acetyl xylan esterase AxeA from *S. lividans* has been reported to be inactive towards chitin.[6] This is not surprising considering that chitin deacetylases from *M. rouxii* and *Colletotrichum lindemuthianum* proved

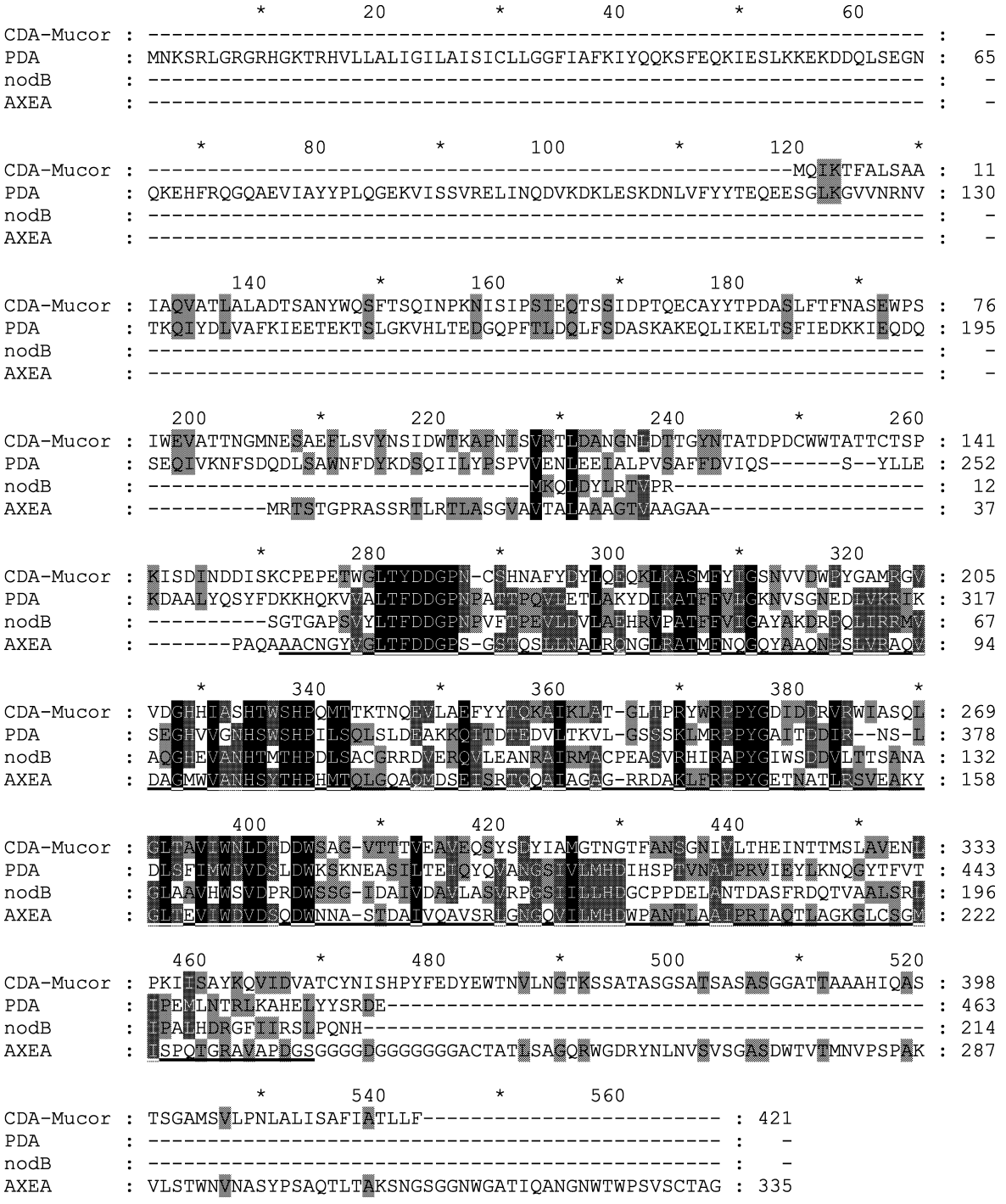


Fig. 2. Multiple sequence alignment of *M. rouxii* CDA (Mucor CDA—Swisprot P50325), peptidoglycan N-acetylglucosamine deacetylase A from *Streptococcus pneumoniae* TIGR4 (PDA-ref NP_345933.1), *Rhizobium sp.* nodB (nodB-pir S34304) and acetyl xylan esterase from *S. lividans* (AxeA-genebank AAC06115.1). The sequence of the truncated AxeA is underlined. The polysaccharide deacetylase domain (NodB domain) has been designated in the Pfam database (accession number PF01522). A related entry can be found under the INTERPRO database (IPR002509).

Table 1
Deacetylation of chitinous substrates and xylan by *M. rouxii* CDA, *S. lividans* AxeA and truncated AxeA

	Glycolchitin (cpm)	Xylan (Acetate released) (nmol)	Chitin-50 (Acetate released) (nmol)	*N*-Acetyl chitotetraose (Acetate released) (nmol)
Mucor CDA	3077	181	84.7	10.4
Mucor CDA + Co^{2+}	4460	101.4	143.9	30
AxeA	92	321.7	118.5	103.8
AxeA + Co^{2+}	400	319.4	635.2	649.2
trAxeA	200	327.5	59.4	68
trAxeA + Co^{2+}	830	311.4	474.3	448.6

The final concentration of birchwood xylan, *N*-acetylchitotetraose, chitin-50 and glycolchitin were 0.75, 1, 0.5 and 0.1 mg/mL, respectively. Reactions were performed using 0.5 mM *M. rouxii* CDA, 1.2 mM *S. lividans* AxeA and 2 mM truncated *S. lividans* AxeA (trAxeA). All reactions involving glycolchitin and peptidoglycan were performed at 50 °C. Reactions involving all other substrates were performed at 37 °C. Co^{2+} was used as chloride at a concentration of 1 mM. Assay conditions are described Section 1.

also to be relatively ineffective towards insoluble chitin substrates.[7,8]

In the presence of Co^{2+} tested as the chloride salt all enzymes examined exhibited increased activity on all soluble chitinous substrates. However, the presence of this metal ion did not result in enhancing the activities of the enzymes when xylan was used as a substrate (Table 1). Chitin deacetylase from *C. lindemuthianum* has been previously reported to be activated by Co^{2+}.[7] Furthermore, deglycosylation of Cda2p from *Saccharomyces cerevisiae* results in total loss of enzyme activity which can be restored by the addition of $CoCl_2$.[9] Further studies are necessary in order to understand this activation process.

The effectiveness of an acetyl xylan esterase from *Bacillus pumilus*,[10] classified in Carbohydrate Esterase Family 7, lacking the NodB homology domain, to deacetylate the chitinous substrates employed in this study was also examined due to the reported broad substrate specificity of this enzyme. However, the enzyme proved to be ineffective in deacetylating chitinous substrates (results not shown).

Finally, chitin deacetylases and both forms of acetyl xylan esterases were inactive when peptidoglycan was employed as substrate. This could be due to the crosslinking of the polysaccharide backbone of peptidoglycan resulting in inefficient enzyme substrate encounters.

We have been unable to perform kinetic studies since most of the substrates used e.g., radiolabelled glycolchitin and peptidoglycan, chitin-50 are not well characterized and their chemical structure varies depending on the experimental conditions employed each time for their preparation.

In this study, it has been demonstrated for the first time that chitin deacetylase from *M. rouxii*, in addition to the presently known soluble chitin substrates examined, is also active on xylan. Furthermore, the acetyl xylan esterase AxeA from *S. lividans* has been shown to be active on various soluble chitin substrates. Finally all enzymes examined were not active on the peptidoglycan substrates examined.

The present work opens up possibilities for tailoring of specificity of Carbohydrate Esterase family 4 enzymes for novel applications.

1. Experimental

1.1. Materials

Glycol chitosan, *N*-acetylchitooligosaccharides (monomer to hexamer) and birchwood xylan were purchased from Sigma. [^{3}H]Acetic anhydride was obtained from DuPont/New England Nuclear. Enzymes and reagents for acetate determination were purchased from Boehringer Mannheim Biochemica. All chromatography media and molecular weight markers were obtained from Pharmacia. Chitin 50 was a gift of Dr K.M. Vårum (Norwegian Biopolymer Laboratory, Institute of Biotechnology, Trondheim, Norway). Chitin-50 presents a mean M_r of 160,000 and is a water-soluble and highly N-acetylated chitosan with a degree of acetylation of 60%, having a Bernoullian distribution of acetylated and deacetylated units. All other chemicals were of the highest purity commercially available. Acetyl Xylan Esterase from *Bacillus pumilis*[10] was a gift from Dr. Degrassi (ICGEB, Trieste, Italy). Purified cell wall peptidoglycan from *B. subtilis* 168 (HF treated)[17] was donated by Professor Simon Foster (University of Sheffield, UK).

1.2. Purification of chitin deacetylase (CDA) from *M. rouxii*

M. rouxii (ATCC no. 24905) CDA (SWISS-

PROT:P50325) was purified to homogeneity according to Kafetzopoulos and co-workers.[11]

1.3. Cloning of $axeA_{tr}$ gene

The gene encoding the truncated form of AxeA was constructed by PCR[12] using plasmid pIAF44 as template[6] and the following primers: axeA5: 5′-CACTCGCATGCGTACCAGTACCGGA-3′; and axeA3-2:5′-CCCGAGCTCTCAACTGCCTCGGGAG-CGACCGC-3′.

All amplifications were performed using a GeneATAQ controller (LKB-Pharmacia) in 100 μL reaction with 1 ng of template, 50 pmoles of each primer, 4% propionamide, 0.05% Tween 20, 20 nmoles of each deoxynucleotides and 2.5 U of *Pfu* polymerase (Stratagene) and the reaction buffer supplied by the manufacturer. The first PCR cycle consisted of a denaturing step at 95 °C for 5 min, an annealing step at 55 °C for 5 min and a polymerisation step at 72 °C for 3 min. Then, DNA was amplified by 30 cycles: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min followed by an extension of the unfinished products at 72 °C for 7 min. A sample (10 μL) was analyzed by agarose gel electrophoresis and the amplicons were recovered using the High Pure PCR Product Purification Kit (Boehringer Mannheim). Protoplasts of *S. lividans* 10–164 (*msi*K^-)[13] were transformed and regenerated on R5 medium.[14] Transformants were grown and kept on Bennett-thiostrepton plates. All constructions were verified by sequencing using the ALF automatic sequencer (Pharmacia Biotech).

1.4. Production and purification of AxeA and $AxeA_{tr}$

Seven days old cultures of *S. lividans* from Bennett-thiostrepton plates were used as initial inoculum. The spores were scraped from the plates and inoculated into 12.5 mL minimal M14 medium (composition per liter: xylose 10g; K_2HPO_4, 5.0 g; $(NH)_4SO_4$, 1.4 g; KH_2PO_4, 1.0 g; $CaCl_2$, 300 mg; $MgSO_4$, 300 mg; $FeSO_4{\cdot}H_2O$, 5.0 mg; $CoCl_2{\cdot}6H_2O$, 2.0 mg; $MnSO_4{\cdot}H_2O$, 1.6 mg; $ZnSO_4{\cdot}7H_2O$, 1.4 mg; Tween 80, 2.0 mL; pH 7.3) and incubated for 18 h at 34 °C with agitation. Bacteria were recovered by centrifugation, used to inoculate 500 mL of the same medium and allowed to grow for 72 h under the same conditions. The supernatant of *S. lividans* culture was first concentrated by ultrafiltration using a 3 kDa cut-off membrane (Omega) and sample (200 mg) was subsequently dialysed against MES-NaOH 20 mM pH 6.0 and applied on a column CM-Sepharose AP2 (Waters, 20×100 mm) equilibrated with the same buffer. Bound proteins were eluted with a linear gradient of NaCl and the active fractions collected and dialysed against 100 mM sodium phosphate pH 6.0 and subsequently loaded on a Superdex HR75 beaded column (3×60 cm; Pharmacia) equilibrated with the same buffer. Purified enzyme containing fractions were pooled, dialysed against water and freeze dried.

1.5. Enzyme activity assays

For the determination of acetate released, we have employed: (a) a radiometric assay using as substrate partially O-hydroxyethylated chitin (glycol chitin), radiolabeled in *N*-acetyl groups. The substrate was prepared according to Araki and Ito.[15] Enzyme assays were performed as previously described[11] in 25 mM sodium glutamate (pH 4.5) buffer for chitin deacetylase from *M. rouxii*, and in 25 mM Tris–HCl (pH 7.5) for Acetyl Xylan Esterase (AxeA) and truncated Acetyl Xylan Esterase (trAxeA) from *S. lividans*. Reactions were performed for 1 h at 50 °C in a 50 μL reaction volume in the presence or absence of 1 mM $CoCl_2$. (b) An enzymatic assay for the determination of acetate released by the action of chitin deacetylase, acetyl xylan esterase AxeA or truncated acetyl xylan esterase on various natural chitinous substrates according to Bergmeyer[16] via three coupled enzyme reactions. The reaction was terminated by heating at 100 °C prior to acetate determination.[8,16]

The assay buffers used were the same as employed for the radioactive labeled substrates. The temperature employed varied depending on the substrate and enzyme used (Table 1).

1.6. Preparation of substrates

Partially O-hydroxyethylated chitin, designated glycol-chitin was prepared according to Araki and Ito.[15]

Preparation of cell wall peptidoglycan from *Bacillus cereus* LMG6910 (BCCM/LMG Bacteria collection, University of Ghent, Belgium) was performed according to Atrih and co-workers,[17] replacing only HF treatment by HCl treatment.

Labeling of cell wall peptidoglycan from *B. cereus* LMG6910 and *B. subtilis* 168 was performed similarly to a method used for labeling glycol chitin.[15] After labeling, the reaction mixture was centrifuged and the supernatant containing soluble peptidoglycan was stored at -20 °C.

Birchwood xylan was acetylated according to Johnson and co-workers[18] using a modified procedure of Dupont and co-workers.[6] For this purpose, xylan was dissolved in Me_2SO at 55 °C and solid potassium borate was added to a final concentration of 0.8%. Acetic anhydride, preheated at 60 °C, was added slowly to the mixture and incubated at room temperature for 2 h. Dimethylsulphoxide and excess acetic acid were removed by extensive dialysis for 5 days against running

tap water, followed by two dialysis steps, 24 h each, against Milli-Q-water. After labelling, the reaction mixture was centrifuged and the supernatant containing soluble acetylated xylan was stored at −20 °C.

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References

1. Coutinho, P.M.; Henrissat, B. **2002**. Carbohydrate-Active Enzymes server at URL: http://afmb.cnrs-mrs.fr/CAZY/.
2. Coutinho, P.M.; Henrissat, B. In *Carbohydrate-active enzymes: an integrated database approach*; Gilbert, H.J.; Davies, G.; Henrissat, B.; Svensson, B., Eds.; Recent advances in carbohydrate bioengineering, The Royal Society of Chemistry, Cambridge, **1999**, pp. 3–12.
3. Kafetzopoulos, D.; Thireos, G.; Vournakis, J. N.; Bouriotis, V. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 8005–8008.
4. Laurie, J. I.; Clarke, J. H.; Ciruela, A.; Faulds, C. B.; Williamson, G.; Gilbert, H. J.; Rixon, J. E.; Millward-Sadler, J.; Hazlewood, G. P. *FEMS Microbiol. Lett.* **1997**, *148*, 261–264.
5. Carmen Limon, M.; Margolles-Cark, E.; Benitez, T.; Penttila, M. *FEMS Microbiol. Lett.* **2001**, 57–63.
6. Dupont, C.; Daigneault, N.; Shareck, F.; Morosoli, R.; Kluepfel, D. *Biochem. J.* **1996**, *319*, 881–886.
7. Tsigos, I.; Bouriotis, V. *J. Biol. Chem.* **1995**, *270*, 26286–26291.
8. Martinou, A.; Kafetzopoulos, D.; Bouriotis, V. *Carbohydr. Res.* **1995**, *273*, 235–242.
9. Martinou, A.; Koutsioulis, D.; Bouriotis, V. *Protein Expr. Purif.* **2002**, 111–116.
10. Degrassi, G.; Kojic, M.; Ljubijankic, G.; Venturi, V. *Microbiology* **2000**, *146*, 1585–1591.
11. Kafetzopoulos, D.; Martinou, A.; Bouriotis, V. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 2564–2568.
12. Yon, J.; Fried, M. *Nucl. Acid Res.* **1989**, *17* (12), 4895.
13. Hurtubise, Y.; Shareck, F.; Kluepfel, D.; Morosoli, R. *Mol. Microb.* **1995**, *17* (2), 367–377.
14. Hopwood, D. A.; Lydiate, D. J.; Malpartida, F.; Wright, H. M. *Bas. Life Sci.* **1985**, *30*, 615–634.
15. Araki, Y.; Ito, E. *Eur. J. Biochem.* **1975**, *55*, 71–78.
16. Bergmeyer, H. U. *Methods Enzyme Anal.* **1974**, *1*, 112–117.
17. Atrih, A.; Bacher, G.; Allmaier, G.; Williamson, M. P.; Foster, S. J. *J. Biol. Chem.* **1999**, *181*, 3956–3966.
18. Johnson, K. G.; Fontana, J. D.; MacKenzie, C. R. *Methods Enzymol.* **1988**, *160*, 551–560.
19. Mergaert, P.; Van Montagu, M.; Holsters, M. *Mol. Microbiol.* **1997**, *25*, 811–817.
20. Cullimore, J. V.; Ranjeva, R.; Bono, J.-J. *Trends Plant Sci.* **2001**, *6*, 24–30.