Molecular Microbiology (2000) **38**(3), 504–513

The *dppA* gene of *Bacillus subtilis* encodes a new D-aminopeptidase

Abdelatif Cheggour,[1†] Laurence Fanuel,[1†] Colette Duez,[1] Bernard Joris,[1] Fabrice Bouillenne,[1] Bart Devreese,[2] Gonzales Van Driessche,[2] Jozef Van Beeumen,[2] Jean-Marie Frère[1] and Colette Goffin[1*]
[1]Centre for Protein Engineering, University of Liège, Institut de Chimie B6, Sart Tilman, 4000 Liège, Belgium.
[2]Laboratorium voor Eiwitbiochemie en Eiwitengineering, Fysiologie en Microbiologie, Universiteit Gent, 9000 Gent, Belgium.

Summary

Different strains of *Bacillus* were screened for their ability to hydrolyse D-alanyl-p-nitroanilide. Activity was detected in *Bacillus pumilus*, *Bacillus brevis*, *Bacillus licheniformis* 749I and *Bacillus subtilis* 168. The last strain was the best producer and was selected for the production and purification of the enzyme. The determination of the N-terminal sequence identified the enzyme as the product of the *dppA* gene (previously named *dciAA*) belonging to the dipeptide ABC transport (*dpp*) operon expressed early during sporulation. Open reading frames (ORFs) encoding putative related proteins were found in the genomes of a variety of Archaea and both sporulating and non-sporulating bacteria. The enzyme behaves as a D-aminopeptidase and represents the prototype of a new peptidase family. Among the tested substrates, the highest activities were found with D-Ala-D-Ala and D-Ala-Gly-Gly. The active enzyme behaves as an octamer of identical 30 kDa subunits. It exhibits a broad pH optimum, extending between pH 9 and 11. It is reversibly inhibited in the presence of Zn^{2+} chelators, and the sequence comparisons highlight the conservation of potential Zn-binding residues. As it has been shown by others that null mutations in the *dpp* operon do not inhibit spore formation, the physiological role of DppA is probably an adaptation to nutrient deficiency.

Introduction

Stereospecific peptidases that recognize the amide bond involving one or two D-amino acid residues are mainly associated with the biosynthesis and remodelling of bacterial wall peptidoglycan. Several of these LD- and DD-carboxy- and transpeptidases have been studied in detail (for reviews, see Frère *et al*., 1992; Holtje, 1998). Most DD-peptidases are active-site serine enzymes, whose active serine also reacts with the β-lactam ring of penicillins to form a relatively long-lived penicilloyl enzyme. In consequence, these DD-peptidases are also called penicillin-binding proteins or PBPs (Goffin and Ghuysen, 1998).

Conversely, a penicillin-insensitive DD-carboxypeptidase secreted by *Streptomyces albus* G (Dpd) was found to be a metallo(Zn^{2+})protein (Dideberg *et al*., 1982). Another unusual DD-carboxypeptidase is VanY, an essential component of the vancomycin resistance system (Arthur *et al*., 1998), whose activity is also dependent on divalent cations. It hydrolyses N^{α}-acetyl-L-Lys-D-Ala-D-Ala and, less efficiently, N^{α}-acetyl-L-Lys-D-Ala-D-lactate. VanX, another essential component of the vancomycin resistance system, hydrolyses D-alanyl-D-alanine, thus preventing its incorporation in the peptidoglycan precursor (Reynolds *et al*., 1994). VanX, VanY and Dpd would belong to a new Zn^{2+} protease family characterized by the presence of the SXHXXGXAXD and EXXH motifs. The VanX and Dpd folds are similar, probably resulting from convergent evolution (Lessard and Walsh, 1999a). Curiously, a VanX homologue (36% identity) is present in the periplasmic space of *Salmonella enterica*. This enzyme exhibits a similar substrate specificity and hydrolyses $(D\text{-}Ala)_2$, but not $(L\text{-}Ala)_2$ or $(D\text{-}Ala)_3$ (Hilbert *et al*., 1999).

Only a few D-amidohydrolases have been described that do not specifically release a C-terminal residue. Four enzymes were isolated by Asano and colleagues from soil microorganisms in a search for enzymes potentially useful in the synthesis of D-amino acid derivatives (Kato *et al*., 1989). The first one is specific for amides of D-amino acids with bulky side-chains (Asano *et al*., 1989a). The second, DAP, is specific for peptides exhibiting an N-terminal D-residue. Its best substrates are D-alanyl derivatives [D-alanyl-amide, $(D\text{-}Ala)_3$, $(D\text{-}Ala)_4$ and D-Ala-L-Ala-L-Ala, whereas $(D\text{-}Ala)_2$ is hydrolysed less efficiently; Asano *et al*., 1989b; 1992]. The third enzyme is an alkaline D-stereospecific endopeptidase (ADP), discovered in screening for $(D\text{-}Phe)_4$-degrading enzymes. It hydrolyses $(D\text{-}Phe)_4$ into $(D\text{-}Phe)_2$, and D-Tyr-D-Phe-D-Phe into D-phenylalanine and D-Tyr-D-Phe (Asano *et al*., 1996). The

Accepted 21 July, 2000. †The first two authors contributed equally to this work. *For correspondence. E-mail cgoffin@ulg.ac.be; Tel. (+32) 4 366 3397; Fax (+32) 4 366 3364.

fourth enzyme, DaaA is a strict D-amino acid amidase devoid of activity on di- and tripeptides. The last three enzymes, DAP, DaaA and ADP, present 27%, 29% and 35% identical residues, respectively, to the penicillin-sensitive DD-carboxypeptidase from *Streptomyces* R61 and are thus related to the PBP family.

Finally, DmpA, an N-terminal nucleophile hydrolase, exhibits different stereospecificities according to the nature of the substrates. It hydrolyses D-alanyl-amide, -p-nitroanilide and -methylester more efficiently than their L-counterparts but is a strict L-aminopeptidase with peptides (Fanuel *et al*., 1999).

Among all these enzymes, only Asano *et al*.'s first two enzymes can strictly be considered as D-aminopeptidases. In order to find new D-aminopeptidases, various strains of *Bacillus* were screened with the chromogenic compound D-alanyl-paranitroanilide (D-Ala-pNa). In this paper, we describe the cloning and some properties of a new D-alanyl-aminopeptidase from *Bacillus subtilis* 168. This protein is not homologous to any of the enzymes mentioned above and is encoded by the first open reading frame (ORF) of the dipeptide transport operon, a gene called *dppA* whose product has not been biochemically characterized before.

Results

Production and purification of the enzyme

Hydrolytic activity versus D-Ala-p-Na was detected in culture supernatants of *Bacillus pumilus*, *Bacillus brevis*, *Bacillus licheniformis* 749I and *B. subtilis* 168. The last strain was the best producer and was therefore selected for further study, another advantage being that the complete sequence of the *B. subtilis* chromosome is known. Various culture media were tested, and activity was determined in the culture supernatants and in cell sonicates. In all cases, the appearance of activity in the culture supernatants was correlated with a strong decrease in the A_{600} value, corresponding to cell lysis of ageing cultures. The highest activity was obtained after 60–80 h of culture in an optimized medium (medium C; see *Experimental procedures*). After the final non-denaturing PAGE, enzymatic activity was located by overlaying the gel with a Whatman paper previously soaked in 10 mM D-Ala-p-Na. The corresponding protein (about 80 μg) was eluted from the gel; its M_r was estimated to be 31 000 by SDS–PAGE and its isoelectric pH to be 5.0. A sample of 200 pmol of enzyme was submitted to N-terminal sequencing, yielding the following result: MKLYMSVDMEGISG–. Exploration of the *B. subtilis* database revealed that this sequence was identical to that of the protein encoded by the *dppA* gene (Mathiopoulos *et al*., 1991). Both the theoretical M_r and isoelectric pH of DppA (30 184 and 5.12 respectively) were in good agreement with those determined for the D-Ala-p-Na-hydrolysing enzyme.

Cloning of the gene and production of the recombinant protein in Escherichia coli

Only the coding region of the *dppA* gene was amplified by polymerase chain reaction (PCR) from genomic DNA of *B. subtilis*, with the two primers described in *Experimental procedures*. The PCR product with a size of about 850 bp corresponded to the structural *dppA* gene with, at the 5′ end, the *Asp*718 and *Nde*I restriction sites and, at the 3′ end, a *Sac*I restriction site. It was subcloned in pUC19 between the *Asp*718 and *Sac*I sites. Its coding sequence was identical to that of the *dppA* gene, with a single discrepancy resulting in the Pro199Ala substitution, in agreement with more recent nucleotide sequence data (Kunst *et al*., 1997). The PCR product was then subcloned in the expression plasmids pET28a and pET22bK^r between the *Nde*I and *Sac*I sites, yielding plasmids pDML1111 and pDML1112, respectively, which were used to transform *E. coli* BL21(DE3) cells.

When carried out at 37°C, the overproduction attempts resulted in the formation of inclusion bodies. Soluble and active enzyme was obtained in the cytoplasm by cooling the culture to 18°C just before induction performed at an A_{600} value of 0.8 by 1 mM IPTG and further growth of the cells at the same temperature for 24 h.

The His_6-tagged protein overproduced with the pDML1111 plasmid was purified on a Ni^{2+}-NTA agarose column. About 2 mg of enzyme was obtained, and an electrospray ionization mass spectrometry (ESMS) analysis revealed the presence of two distinct species exhibiting M_r values of 30 909 ± 4.4 (60%) and 30 444 ± 3.6 (40%), well below the expected value of 32 322. The determination of the C-terminal sequence (three residues) gave the expected result, thus excluding the action of a carboxypeptidase. The N-terminal sequence revealed a heterogeneity with a loss of 13 or 17 residues from the N-terminal extension as a result of the genetic construction. The theoretical M_r values for these truncated recombinant proteins, 30 906 and 30 440, respectively, were in good agreement with those measured by ESMS. In both cases, the six histidines had been eliminated. The k_{cat}/K_m value of 80 000 M^{-1} s^{-1} was determined with the heterogeneous protein and 120 μM D-Ala-pNa at a concentration well below the K_m value (see the next section).

To avoid the N-terminal heterogeneity, PDML1112 was used to overproduce a protein identical to that found in the original *B. subtilis* strain. The purification was performed as described in *Experimental procedures*. Owing to the rather low capacity of the MonoQ column, the last step was repeated several times, each run yielding about 1.2 mg of enzyme exhibiting a purity of more than 95%. Fifty milligrams of pure enzyme was thus obtained starting from 1 l of culture. The enzyme solution was dialysed

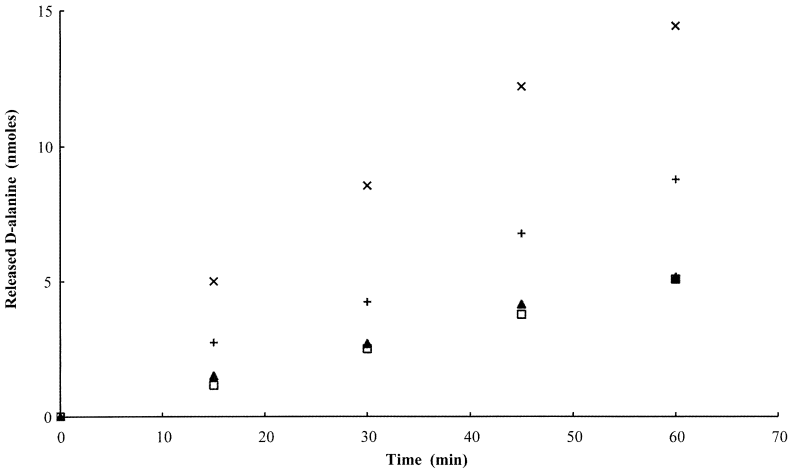

Fig. 1 Time courses of the hydrolysis of D-alanyl-R oligopeptides by DppA. Samples of 0.8 μmol of (D-Ala)$_2$ (**X**), D-Ala-Gly-Gly (+), (D-Ala)$_3$ (□) or (D-Ala)$_4$ (▲) were incubated at 30°C with 0.28 nmol of pure DppA in a total volume of 100 μl of buffer B with 0.1% BSA added. The released D-alanine was quantified by the D-amino acid oxidase assay (Frère *et al.*, 1976). SD values did not exceed 10%. With D-Ala-Gly and under the same conditions, about 2 nmol of D-alanine was detected after 1 h.

against 50 mM KPi, pH 8 (buffer A), concentrated to 3.5 mg ml^{-1} for preliminary characterization and kinetic studies, or to 15 mg ml^{-1} for crystallographic studies, and stored at −20°C. The M_r value determined by ESMS was 30 152.7 ± 6, in good agreement with that calculated from the sequence (30 158.7).

Characterization of the protein

The hydrolysis of 120 μM D-Ala-p-Na remained strictly first order throughout complete hydrolysis time courses, from which a k_{cat}/K_m value of 100 000 ± 10 000 M^{-1} s^{-1} was deduced. Accordingly, the rate of hydrolysis of the same substrate remained proportional to its concentration up to 1 mM, indicating that the K_m value was significantly higher than the latter concentration. With 1 mM D-Ala-pNa in 50 mM KPi, pH 8, with 40 μM $ZnSO_4$ added (buffer B), the specific activity of the enzyme was 80 μmol min^{-1} (IU) mg^{-1} pure protein.

Dialysis of the purified preparation (116 μM) against 50 mM HEPES buffer, pH 8.0, resulted in a significant decrease in activity, but the addition of Zn^{2+} concentrations > 10 μM to diluted samples restored the initial activity. Other metal cations (Fe^{3+}, Mn^{2+}, Cu^{2+} and Co^{2+}) were less efficient or showed no effect. The activity was inhibited as expected in the presence of 8 mM 1, 10-phenanthroline or 50 mM EDTA.

The activity also decreased in the presence of monovalent cations. Residual activities of 80%, 40% and 15% were found in the presence of 0.125, 1.0 and 4.0 M NaCl respectively. Similar results were obtained with KCl.

After 2 h at 50°C or 10 min at 60°C, the specific activity remained unchanged, but decreased to 20% after 10 min at 75°C. Incubation of 1 nM enzyme for 120 min or of 120 nM for 1 min at 30°C with 120 μM D-Ala-pNa yielded the same quantity of product (Selwyn's test), indicating that the enzyme was stable for at least 2 h at the lowest concentration in the presence of a substrate concentration well below the K_m value.

Finally, the enzyme exhibited a rather broad optimum in the pH range 9–11. At pH values of 8, 7 and 6, the activities corresponded to 60%, 20% and 2% of the maximum respectively.

DppA activity on oligopeptides – qualitative analysis

To characterize the catalytic specificity of DppA further, various oligopeptides containing L- or D-amino acid residues were tested as possible substrates. The products were analysed by thin-layer chromatography (TLC) on silica gel plates.

After a 24 h incubation with 11.6 μM pure DppA (specific activity versus D-Ala-pNa, 80 IU mg^{-1}) at 30°C, no hydrolysis was observed with the following peptides at concentrations of 18 or 9 mM (see *Experimental procedures*): (L-Ala)$_2$, L-Ala-D-Ala, L-Ala-Gly, Gly-L-Leu, L-Arg-L-Phe, (L-Phe)$_2$, (L-Phe)$_3$, L-Phe-Gly-Gly, (L-Ser)$_3$, L-Ala-Gly-Gly, (Gly)$_3$, (Gly)$_4$, (Gly)$_5$. Only faint spots corresponding to D-Ala and/or L-Ala were observed upon incubation of D-Ala-L-Ala, L-Ala-L-Ala or (L-Ala)$_4$ under the same conditions. In contrast, the D-alanine release from D-Ala-D-Ala and D-Ala-Gly-Gly was complete after a 2 h incubation. In the second case, the resulting Gly-Gly dipeptide was only partially hydrolysed after a much longer incubation (24 h).

Activity on oligopeptides – quantitative analysis

The release of D-alanine from D-Ala-D-Ala, (D-Ala)$_3$, (D-Ala)$_4$, D-Ala-Gly and D-Ala-Gly-Gly, monitored with the help of the D-amino acid oxidase test, revealed that D-Ala-D-Ala and D-Ala-Gly-Gly were the best substrates (Fig. 1) with turnover numbers of about 5 min^{-1} (at an 8 mM substrate concentration).

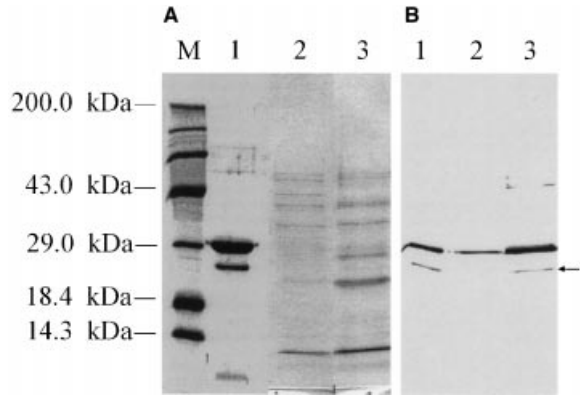

Fig. 2. SDS–PAGE analysis of purified DppA and of culture supernatants of *B. subtilis.*
A. Coomassie blue staining.
B. Western blot.Lane M, molecular mass standards; lanes 1A and 1B, DppA purified from *E. coli*: 5 μg (A) or 10 ng, i.e. 0.8 mIU (B); lanes 2A and 2B, 20 μl of sevenfold concentrated supernatant from a 36 h *B. subtilis* culture (about 1 μg of total protein and 0.08 mIU); lanes 3A and 3B,15 μl of supernatant from a 72 h *B. subtilis* culture (about 2 μg of total protein and 1.2 mIU).The arrow indicates the position of the 24 kDa polypeptide (see text).

Molecular properties

After electrophoresis on a 7.5% non-denaturing polyacrylamide gel, Coomassie blue staining revealed one single protein band, but the migration velocity appeared to be very low for a 30 kDa protein with an isoelectric pH of 5.1. Accordingly, chromatography on an analytical Superdex 200 column in buffer B indicated an M_r value of 230 ± 15 kDa. In consequence, the native enzyme might be an octamer.

SDS–PAGE analysis revealed one major band with the expected M_r of about 30 kDa, but also two minor bands (Fig. 2A, lane 1). The larger one, with a slightly lower M_r than that of the major protein, represented at most 5–10% of the amount of the latter, and the smaller one exhibited a M_r value of about 6 kDa. The N-terminal sequences of the three peptides were determined. The major protein and the 6 kDa peptide had the same 10 N-terminal residues. The N-terminal sequence of the larger minor peptide corresponded to an internal sequence beginning at Ser-61. As the active enzyme is a multimeric protein that migrates in a non-denaturing PAGE as a homogeneous single species, it can be concluded that the two polypeptide chains generated by the His-60/Ser-61 cleavage remain associated and retain the same folding.

Western blot analysis

To study the protein produced by the original strain of *B. subtilis*, samples of supernatants from 36 h and 72 h cultures were analysed by Western blotting after SDS–PAGE. The results are shown in Fig. 2.

Coomassie blue staining of the gel revealed, as expected, that many proteins were produced in large quantities (Fig. 2A, lanes 2 and 3), but the amounts of DppA estimated by measuring the activity with 1 mM D-Ala-pNa were about 1 and 15 ng in lanes 2 and 3, respectively, and thus well below the detection limit of Coomassie blue staining.

The Western blot analysis revealed two bands both in a 10 ng sample of purified DppA (produced in *E. coli*) and in the 72 h *B. subtilis* culture supernatant (Fig. 2B, lane 1 and 3 respectively). In the former, the positions and intensities of the two bands corresponded to those of the 30 kDa and 24 kDa peptides visualized by Coomassie blue staining and, in the latter, the major species remained the 30 kDa protein, but a smaller peptide of about 24 kDa was visible (Fig. 2B, arrow). In contrast, in the 36 h culture supernatant, the Western blot revealed a single, but much less intense, 30 kDa DppA species, so that the smaller peptide might be present but remain undetectable. So, when produced by the original strain, a small amount of the 30 kDa DppA was also cleaved, and the sizes of the fragments were similar to those observed with the enzyme produced with *E. coli.*

Alignment analysis

An exploration of the databases revealed 11 sequences related to the DppA sequence. Figure 3 shows the multiple sequence alignments. The similarity between the 12 sequences is particularly significant in the first 200 amino acid residues with several highly conserved residues, even when one includes the much shorter (incomplete?) *Bordetella* sequences. Among the strictly conserved residues, Asp-8, Glu-10 and His-60 could be involved in the interactions with Zn^{2+} ions, although these conclusions might be questioned if the 24 kDa form was found to be active. It is interesting to note that a DppA-like enzyme seems to exist in very different genera (Gram+, Gram–, *Streptomyces*, *Archaea*), both sporulating (*Bacillus*, *Clostridium*,…) and non-sporulating (*Deinococcus*, *Archaea*).

Discussion

While screening different bacterial species for their ability to hydrolyse D-alanyl-para-nitroanilide, we found that several members of the *Bacillus* genus produced such an activity, which was later shown to be associated with a D-aminopeptidase. The enzyme was purified from *B. subtilis*; the corresponding gene was cloned, sequenced and found to be *dppA*, the first ORF of the *dpp* operon that is expressed early during sporulation according to Mathiopoulos *et al*. (1991). To facilitate the purification, the enzyme was first produced with an N-terminal extension containing a $(His)_6$ tag. However, this resulted in the isolation of two proteolytic products in which only

Fig. 3. Alignment of the DppA sequence (*B. subtilis*) with those of related sequences found in the databases. (*) indicates conserved residues, (:) conservative substitutions and (.) semi-conservative substitutions in all sequences excluding the incomplete *Bordetella pertussis* and *B. bronchiseptica* sequences. The heavy arrow indicates the cleavage site observed in DppA purified from *E. coli* (see text). The light arrows indicate the conserved residues that might be involved in the interaction with Zn^{2+} ions.

parts of the extension had been retained. The k_{cat}/K_m value versus D-Ala-pNa measured with this heterogeneous preparation was 80 000 M^{-1} s^{-1}. Thus, these N-terminal alterations have little effect on D-Ala-pNa hydrolysis. As already mentioned in the literature (Ledent *et al*., 1997), this type of protein engineering, although potentially simplifying the purification procedure, may have some unexpected consequences on the biochemical characteristics of the target protein and must be used with care. In consequence, a protein identical to the original *B. subtilis* one was produced in *E. coli.*

Molecular sieve chromatography suggested that the active enzyme was an octamer composed of identical subunits. In contrast to the D-aminopeptidase from *Ochrobactrum anthropi* (DAP), DppA does not hydrolyse D-Ala-L-Ala and has a poor efficiency on peptide substrates such as D-Ala-Gly-Gly. VanX and the periplasmic D-alanyl-D-alanine dipeptidase from *Salmonella enterica* do not hydrolyse $(D\text{-Ala})_3$, one of the good substrates of DppA. DmpA, the L-aminopeptidase D-Ala-esterase/amidase from *O. anthropi* hydrolyses L-Ala-pNa and various L-dipeptides, whereas DppA is devoid of activity versus L-derivates and behaves as a strict D-stereospecific amidohydrolase far more active on D-Ala-pNa ($k_{cat}/K_m = 100\,000$ M^{-1} s^{-1} with a k_{cat} value of at least 100 s^{-1} as K_m is >1 mM) than on its best peptide substrates $(D\text{-Ala})_2$ and D-Ala-Gly-Gly (turnover numbers of about 5 min^{-1}). With D-Ala-pNa, the pH dependence of DppA is bell-shaped with a broad maximum extending from pH 9 to pH 11. A 90% inhibition was observed in the presence of Zn^{2+} chelators. The initial activity could be recovered by the addition of 10 μM Zn^{2+}.

An exploration of the databases showed that the genomes of *Bacillus methanolicus*, *Deinococcus radiodurans and Streptomyces coelicolor* contained ORFs encoding putative proteins very similar to DppA. Other genomes mentioned in Fig. 3 (*Pyrococcus abysii*, *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Aeropyrum pernix*, *Enterococcus faecalis*, *Bordetella pertussis*, *Bordetella bronchiseptica* and *Clostridium difficile*) also contained ORFs encoding putative, probably related proteins. Some residues, often involved in Zn^{2+} binding in metalloenzymes, are conserved in these DppA-related sequences.

To date, none of the DppA-like proteins mentioned above has been biochemically characterized. The amino acid sequences of the known D-aminopeptidases are not related to that of DppA. The catalytic specificity of DppA is original. In consequence, DppA might be the prototype of a new family of D-aminopeptidases, although the exact function of some homologous proteins might have evolved in a somewhat different direction.

The gene encoding DppA was described by Mathiopoulos *et al*. (1991) as the first ORF of the decoyinine-inducible or *dciA* operon. Decoyinine, an inhibitor of GMP synthetase, induces sporulation of *B. subtilis* (Mitani *et al*., 1977). The *dciA* operon is also called the dipeptide transport operon or *dpp* because the four genes that follow the *dciAA/dppA* gene encode the elements of an ATP-binding cassette (ABC) transporter (Kuan *et al*., 1995; Saurin *et al*., 1999), and disruption of the *dciAE* gene renders a proline auxotroph unable to grow on Pro-Gly as sole proline source (Mathiopoulos *et al*., 1991).

The *dpp* operon is negatively regulated by two *trans*-acting factors, AbrB and CodY (control of *dciA/dpp* operon) (Slack *et al*., 1991; 1993; 1995; Serror and Sonenshein, 1996). AbrB is a well-known repressor of some genes expressed early during sporulation (Robertson *et al*., 1989), and CodY would belong to a nutrient-sensing mechanism. CodY competes with AbrB for the promoter region of the *dpp* operon, but the repressor activity of CodY requests the presence of an unidentified co-repressor. Nutritional adaptation and spore formation are usually considered as competitive and mutually exclusive processes (Mathiopoulos *et al*., 1991), but the choice between the two pathways depends on an integration of multiple signals. The identification of the enzymatic reactions catalysed by the first ORF of this *dciA/dpp* operon might contribute to a better understanding of these phenomena.

The physiological role of DppA, a D-aminopeptidase that also hydrolyses $(D\text{-Ala})_2$ and is expressed early during sporulation, is probably an adaptation to nutrient deficiency rather than an element favouring sporulation by hydrolysing the D-Ala-D-Ala dipeptide required in peptidoglycan biosynthesis. Indeed, DppA-like proteins are found in microorganisms that do not sporulate (*Archaea*, *Deinococcus radiodurans*, *Bordetellae*), and Mathiopoulos *et al*. (1991) have already mentioned that null mutations in the *dciA* operon do not inhibit spore formation. The released D-alanine could then be used as a metabolic fuel. A similar role has been proposed for the VanX homologue (DdpX) in *E. coli* (Lessard and Walsh, 1999b). Interestingly, the genes encoding DdpX and the DaaA amidase produced by *O. anthropi* and mentioned in the *Introduction* are also parts of peptide transport operons (Lessard and Walsh, 1999b; Komeda and Asano, 2000).

The present study highlights the complementarity of genome sequencing and biochemical analysis. The availability of the complete *B. subtilis* genome sequence allowed rapid cloning of the gene after very small amounts of the protein were purified.

Experimental procedures

Enzymes, strains, vectors, chemicals, culture media and antibodies

Molecular biology and sequencing kits, purification gels,

columns and Ampholine PAG plates were supplied by Pharmacia Biotech unless otherwise mentioned. The nitrilotriacetic acid (NTA) agarose was obtained from Affiland. The T4 DNA ligase was from Boehringer Mannheim. The restriction enzymes were purchased from Life Technologies. The *E. coli* BL21(DE3) strain and the expression vectors pET28a and pET22b were supplied by Novagen; the ampicillin resistance gene of the pET22b plasmid was inactivated by the insertion of a kanamycin resistance cartridge at the *Pst*I site, and the resulting vector was named pET22bK^r (details of this construction can be obtained from the authors). IPTG was from Eurogentec, substrates and peptides from Bachem and Sigma. The TLC plates (Silica gel 60F250) were from Merck. Rabbit anti-DppA antibodies were obtained from the 'Centre d'Economie Rurale' Marloie, Belgium.

Luria–Bertani medium (10 g of bactotryptone, 5 g of yeast extract and 10 g of NaCl in 1 l of water, adjusted to pH 7.0) was used as a reference. Aliquots (10 ml) of the culture, taken at different times, were centrifuged. The pellets were suspended in 1 ml of fresh culture medium and sonicated. The estimation of D-aminopeptidase activity in the culture supernatants or in sonicated cell extracts was carried out by monitoring the formation of p-nitroaniline from D-Ala-p-Na, at 405 nm and 30°C as described by Asano *et al.* (1992). Various other media were tested. They were prepared by adding glucose, glycerol, sodium lactate, bovine serum albumin (BSA) or casein hydrolysates to a basic medium (BM) described by Park and Reardon (1996) and containing 1 g of yeast extract and 15 g of nutrient broth l^{-1}. The best production was obtained by adding 0.5 g of glucose and 7.2 g of sodium lactate to 1 l of BM, yielding medium C.

Purification of the D-aminopeptidase from B. subtilis

After 70 h of culture at 28°C, 2.5 l of supernatant were recovered and diluted to 10 l with 10 mM potassium phosphate buffer (KPi), pH 6.5. The pH was adjusted to 6.5 by the addition of HCl. Seventy grams of Q Sepharose Fast Flow (QSFF; Pharmacia Biotech) equilibrated in the same buffer was added, and the mixture was stirred at 4°C until complete adsorption of the D-aminopeptidase activity. The loaded exchanger was recovered by filtration, washed extensively with 10 mM KPi, pH 6.5, and eluted twice with 100 ml of 50 mM KPi, pH 6.5, containing 300 mM KCl. Both eluates were pooled, concentrated and dialysed against 50 mM KPi, pH 6.5. The sample was loaded onto a 200 ml QSFF column equilibrated with 50 mM KPi, pH 6.5, and a linear KCl gradient (0–350 mM over 10 column volumes) was applied in the same buffer. The D-aminopeptidase activity was eluted between 250 and 300 mM KCl. The most active fractions were concentrated, dialysed against 50 mM KPi, pH 8 (buffer A), and loaded onto a second QSFF column equilibrated in buffer A. A linear NaCl gradient (100–500 mM) was applied in the same buffer, and the active fractions, containing about 16 mg of impure protein, were eluted between 300 and 400 mM NaCl. After dialysis against buffer A and concentration by ultrafiltration, a 1 mg sample was submitted to electrophoresis on an 8% non-denaturating polyacrylamide gel. The enzymatic activity was located by overlaying a Whatman paper previously soaked in 10 mM D-Ala-p-Na. The protein band corresponding to the yellow area was cut out and eluted at 4°C in 1 ml of buffer A.

During the purification steps, the protein concentration was estimated on the basis of the absorbance at 280 nm.

Isoelectrofocusing (IEF)

The isoelectric pH value was evaluated on Ampholine PAG plates, pH 3.5–9.5, by detecting the active band with D-Ala-p-Na and measuring the pH at its position.

N- and C-terminal sequences

N-terminal sequence analysis was carried out on a pulsed-liquid sequencer with on-line analysis of the PTH amino acids (Perkin-Elmer, Applied Biosystems Division). C-terminal sequencing was performed on a Procise 494C sequencer (Perkin-Elmer) using a slight modification of the protocol described by Boyd *et al.* (1992). The alkylated thiohydantoins were identified by reverse-phase high-performance liquid chromatography (HPLC). Before sequence analysis, the lysine side-chains were modified with phenylisothiocyanate under basic conditions.

Mass spectrometry (ESMS)

ESMS was carried out on a Bio-Q quadrupole mass spectrometer (Micromass). Ten microlitres of a sample solution in 50% acetonitrile−1% formic acid was injected manually in the 20 μl loop of the Rheodyne injector and pumped with a Harvard syringe pump at a flow rate of 5 μl min^{-1}. Scans of 9 s over the mass range of 600–1500 a.m.m. were collected over 2 min. Mass calibration was performed with horse heart cytochrome *c*.

Kinetic measurements

The enzyme activity was measured in buffer A with 40 μM $ZnSO_4$ (buffer B) added in a total volume of 400 μl at 30°C. For substrates containing a p-nitroaniline leaving group, the variation in absorbance was monitored at 405 nm for v_0 measurements ($\Delta\varepsilon = 11\ 500\ M^{-1}\ cm^{-1}$) or at 440 nm ($\Delta\varepsilon = 2250\ M^{-1}\ cm^{-1}$) for complete time course analyses. The k_{cat}/K_m value was obtained by linear regression of the v_0 values or by analysis of the complete time courses with the help of the first-order equation (De Meester *et al.*, 1987). As the active form of DppA is an oligomer (see below), the enzyme concentration is expressed as that of active sites.

TLC analysis

The hydrolysis products of peptides containing L-alanyl or D-alanyl N-terminal residues were separated from residual substrates by TLC on silica gel plates. In order to separate D- or L-alanine from the di-, tri- or tetra-alanyl peptides, the TLC solvent was ethanol–H_2O–triethylamine (70:25:5 by volume). After a 10 cm migration, the plate was dried at room temperature, dipped in the ninhydrin reagent for 5 s and dried at 110°C for 15 min (Gerday *et al.*, 1968).

To separate D- or L-alanine from D- or L-alanyl-glycyl-glycine, glycyl-glycine and glycine, the TLC solvent was *n*-butanol–acetic acid–5% ammonium hydroxide (55:30:15 by volume). After a 20 cm migration, the plate was dried at 110°C for 1 h and treated as above. Scanning of the digital photography was performed using the Cybertech C5-1 system, and the signal intensities were compared with those of known amounts of the various compounds treated in parallel on the same plate.

Recombinant DNA techniques, bacterial strains, plasmids and growth conditions

The procedures used were essentially those described by Sambrook *et al*. (1989).

The genomic DNA of *B. subtilis* 168 (Genetic Stock Centre, USA) was extracted with the Qiagen genomic tip 20/G kit.

The sequences of the oligonucleotides used as primers for the PCR experiments and deduced from that of the *dppA* gene were as follows: 5′-CGGGGTACCTCATATGAAATTG TACATGTCAGTAGATATGGAAGG-3′ and 5′-CCCGAGCT CTTAGCAGAATGATGTCCGCATCGC-3′. The underlined palindromic sequences are recognized by the *Asp*718, *Nde*I and *Sac*I restriction enzymes respectively.

The PCR mixture (100 μl total volume) contained 1.5 μg of DNA template, 0.5 μg of each primer, 2.5 units of Vent polymerase (New England Biolabs) in the buffer recommended by the supplier.

The PCR was carried out with a DNA thermal cycler (Biometra-Trio Thermoblock) for 25 cycles with a 1 min denaturation at 94°C, a 1 min annealing at 50°C and a 1 min polymerization at 72°C. The PCR product was purified using the Geneclean spin kit (Bio 101).

PMDL 1110. The PCR product, digested with the *Asp*718 and *Sac*I enzymes, was ligated in the pUC19 cloning vector previously cut with the same enzymes. The recombinant plasmid (PDML1110) was extracted, purified, and the insert was completely sequenced on both strands with the Auto-Read sequencing kit and the ThermoSequenase labelled primer cycle sequencing kit. Sequences were read on an automated laser fluorescent DNA sequencer.

PDML 1111 and PDML 1112. To overexpress the gene coding for the D-alanyl aminopeptidase activity, PDML 1110 was digested with the *Nde*I and *Sac*I enzymes. The resulting fragment was ligated in pET28a or pET22bK^r, previously cut with the same enzymes, yielding PDML 1111 and PDML 1112 respectively.

Purification of the overproduced D*-alanyl amidases in* E. coli

As a result of cloning the PCR product in pET28a, PDML 1111 encodes a protein with an additional N-terminal 20 residues including six consecutive His. In consequence, the recombinant protein was expected to be retained by a Ni^{2+}-NTA agarose column. Nevertheless, some D-alanyl aminopeptidase activity was not retained. The adsorbed enzyme was eluted with an imidazole linear gradient (0–250 mM) at pH 7.4. The fractions containing the D-alanyl amidase activity were pooled, dialysed against buffer A and concentrated. About 2 mg of enzyme was obtained.

The PDML 1112 plasmid encodes exactly the same protein as that produced by the original *B. subtilis* strain. A 2 l *E. coli* culture in LB medium was grown to an A_{600} value of 0.8 at 37°C, cooled to 18°C and induced by 1 mM IPTG. After a 24 h incubation at 18°C, the cells were harvested by centrifugation. The pellet was suspended in 100 ml of Tris-HCl (10 mM, pH 8), and the cells were disrupted with the constant basic system disintegrator (Inceltech). The suspension was centrifuged, and this crude extract supernatant was dialysed against 50 mM KPi buffer at pH 6.5 and loaded onto a 100 ml Q-Sepharose column equilibrated in the same buffer. After washing, the enzyme was eluted by applying a 0–0.3 M NaCl gradient in the same buffer over 1500 ml. The active fractions were pooled and dialysed against buffer A, then loaded onto a Q-Sepharose column equilibrated with the same buffer. Elution was carried out using a 0–0.5 M NaCl gradient (1.5 l). The active fractions were pooled, concentrated to 50 ml by ultrafiltration and filtered through a 400 ml Superdex G100 column in buffer A. The active fractions were pooled (200 ml), and 5 ml aliquots were loaded onto a MonoQ column (HR 5/5, 1 ml) connected to an Åkta Explorer apparatus (Pharmacia Biotech). After a 20 ml wash, 15 ml linear (0–0.120 M) and 60 ml linear (0.120–0.240 M) NaCl gradients, both in buffer A, were applied successively. Under these conditions, the peak of activity was well separated from minor amounts of contaminant proteins. Forty runs were carried out always presenting the same distribution of protein peaks. The active fractions were pooled, dialysed against buffer A, concentrated and stored at −20°C.

pH dependence of the hydrolysis of D*-Ala-pNa*

The universal buffer system of Teorell and Stenhagen (1938) (pH 2.0–12.0) was prepared with a minor modification. Aliquots of the concentrated mixture of the three acids (citric, phosphoric and boric) were neutralized with NaOH up to the desired pH, then diluted to the same final volumes. The buffered D-Ala-pNa substrate solutions (1.0 mM) were complemented with 40 μM $ZnSO_4$, and the v_0 values were determined.

Thermal stability

The enzyme (11.6 μM) was incubated in buffer B for 10 min at different temperatures. The DppA activity was assayed at 30°C with 1.0 mM D-Ala-pNa.

Activity on peptide substrates

Stock solutions (20 mM) of alanyl peptides [$(L\text{-Ala})_2$, $(D\text{-Ala})_2$, L-Ala-D-Ala, D-Ala-L-Ala, $(L\text{-Ala})_4$, $(D\text{-Ala})_4$] were prepared in buffer B. For all other peptides, the stock solutions were 10 mM in the same buffer.

To 45 μl of peptide solution, 5 μl of 116 μM DppA in buffer B or 5 μl of buffer B were added, and incubation was carried out at 30°C over 2 or 24 h. The reaction was stopped by

heating the samples to 100°C for 2 min. Aliquots (2 μl) were analysed by TLC.

With (D-Ala)$_2$, D-Ala-Gly, (D-Ala)$_3$, D-Ala-Gly-Gly and (D-Ala)$_4$, the released D-alanine was more accurately quantified by the D-amino acid oxidase assay (Frère *et al*., 1976). To 80 μl of 10 mM substrate in buffer B, 20 μl of 11.6 μM DppA containing 1% BSA was added. After various periods of time, the reaction was stopped as above, and the assay was performed on 10 μl aliquots.

Gel electrophoresis

In the presence of SDS with 4% and 15% stacking and running gels, respectively, PAGE was performed as described by Laemmli (1970). Under non-denaturing conditions, 4% and 7.5% stacking and running gels were used respectively. Aliquots of enzyme preparations in buffer B were deposited on the gel, and the protein bands were revealed by Coomassie blue staining.

Analytical molecular sieve

Aliquots (50 μl) of 30 μM or 0.3 μM DppA in buffer B were filtered through a Superdex TM 200 HR 10/30 column equilibrated in the same buffer and connected to the Åkta Explorer. Calibration was performed with the following proteins: thyroglobulin (669 000), apoferritin (443 000), catalase (232 000), L-alanine dehydrogenase (228 000), aldolase (158 000), alcohol dehydrogenase (150 000), apotransferrin (80 000), BSA (66 700), ovalbumin (43 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 000) and lysozyme (14 300).

Western analysis

Rabbit antibodies were raised against the recombinant DppA purified from *E. coli*. Pure Dppa (10 ng) or 1–2 μg of (total) proteins present in the supernatants of 36 h or 72 h *B. subtilis* cultures were submitted to SDS–PAGE. After electroblotting onto a nitrocellulose membrane, the adsorbed proteins were stained with 0.1% Ponceau S (w/v) in 5% acetic acid (v/v) (Sigma) for localizing the standard proteins. Thereafter, the membrane was rinsed with water and treated as described in the Bio-Rad ImmunoBlot manual using goat anti-rabbit–alkaline phosphatase conjugates.

Alignments

Preliminary sequence data were obtained from the Institute for Genomic Research website at http://www.tigr.org. The amino acid sequences of *B. subtilis* DppA and of its putative homologues can be accessed through the following accession numbers in the NCBI Protein Database: *B. subtilis* DppA, no. CAA40002(Mathiopoulos *et al*., 1991); *A. pernix*, no. BAA79269(Kawarabayasi *et al*., 1999); *Bacillus methanolicus*, no. AAB39857(Cue *et al*., 1997); *D. radiodurans*, no. AAF11394(White *et al*., 1999); *P. abyssi*, no. CAB49502; *P. horikoshii*, no. BAA30694(Kawarabayasi *et al*., 1998); and *S. coelicolor*, no. CAA22734(Redenbach *et al*., 1996). Multiple sequence alignments were performed with the CLUSTAL algorithm (website: http://www2.ebi.ac.uk/clustalw/).

Acknowledgements

The authors wish to thank Mrs Nicole Gérardin from the Laboratory of Biochemistry for her useful help in the TLC analysis, Professor Edwin De Pauw and Mr Emmanuel Delye for many useful discussions. Sequencing of *Bordetella bronchiseptica*, *B. pertussis* and *Clostridium difficile* was performed with support from Beowulf Genomics; sequencing of *Enterococcus faecalis* with support from NIAID; sequencing of *Pyrococcus furiosus* with support from DOE; and sequencing of *Streptomyces coelicolor* with support from BBSRC/Beowulf Genomics. This work was supported in part by the Belgian Program of Interuniversity Poles of Attraction (PAI no. P4/03) and a Geconcerteerde Onderzoeks-aktie from the Flemish Government (120.522.93). The purchase of the Åkta Explorer apparatus was made possible by a grant from the Fonds National de la Recherche Scientifique (FNRS, Brussels, Belgium). L.F. and B.J., C.D. and C.G. are, respectively, Aspirant and Chercheurs Qualifiés of the FNRS. B.D. is a postdoctoral researcher from the Fonds voor Wetenschappelijk Onderzoek (FWO, Flanders, Belgium).

References

Arthur, M., Depardieu, F., Cabanie, L., Reynolds, P., and Courvalin, P. (1998) Requirement of the VanY and VanX D,D-peptidases for glycopeptide resistance in enterococci. *Mol Microbiol* **30**: 819–830.

Asano, Y., Mori, T., Hanamoto, S., Kato, Y., and Nakazawa, A. (1989a) A new D-stereospecific amino acid amidase from *Ochrobactrum anthropi*. *Biochem Biophys Res Commun* **162**: 470–474.

Asano, Y., Nakazawa, A., Kato, Y., and Kondo, K. (1989b) Properties of a novel D-stereospecific aminopeptidase from *Ochrobactrum anthropi*. *J Biol Chem* **264**: 14233–14239.

Asano, Y., Kato, Y., Yamada, A., and Kondo, K. (1992) Structural similarity of D-aminopeptidase to carboxypeptidase DD and beta-lactamases. *Biochemistry* **31**: 2316–2328.

Asano, Y., Ito, H., Dairi, T., and Kato, Y. (1996) An alkaline D-stereospecific endopeptidase with beta-lactamase activity from *Bacillus cereus*. *J Biol Chem* **271**: 30256–30262.

Boyd, V.L., Bozzini, M., Zon, G., Noble, R.L., and Mattaliano, R.J. (1992) Sequencing of peptides and proteins from the carboxy terminus. *Anal Biochem* **206**: 344–352.

Cue, D., Lam, H., Dillingham, R.L., Hanson, R.S., and Flickinger, M.C. (1997) Genetic manipulation of *Bacillus methanolicus*, a Gram-positive, thermotolerant methylotroph. *Appl Environ Microbiol* **63**: 1406–1420.

De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frère, J.M., and Waley, S.G. (1987) Automated analysis of enzyme inactivation phenomena. Application to beta-lactamases and DD-peptidases. *Biochem Pharmacol* **36**: 2393–2403.

Dideberg, O., Charlier, P., Dive, G., Joris, B., Frère, J.M., and Ghuysen, J.M. (1982) Structure of a Zn^{2+}-containing D-alanyl-D-alanine-cleaving carboxypeptidase at 2.5 A resolution. *Nature* **299**: 469–470.

Fanuel, L., Goffin, C., Cheggour, A., Devreese, B., Van Driessche, G., Joris, B., *et al*. (1999) The DmpA aminopeptidase from *Ochrobactrum anthropi* LMG7991 is the prototype of a new terminal nucleophile hydrolase family. *Biochem J* **341**: 147–155.

Frère, J.-M., Leyh-Bouille, M., Ghuysen, J.-M., Nieto, M., and Perkins, H.R. (1976) Exocellular DD-carboxypeptidases-transpeptidases from *Streptomyces.* In *Methods in Enzymology. Proteolytic Enzymes*, Part B, XLV. Lorand, L. (ed.). New York: Academic Press, pp. 610–636.

Frère, J.-M., Nguyen-Distèche, M., Coyette, J., and Joris, B. (1992) Mode of action: interaction with the penicillin binding proteins. In *The Chemistry of* β*-Lactams.* Page, M.I. (ed.). Glasgow: Blackie, pp. 149–198.

Gerday, C., Robyns, E., and Gosselin-Rey, C. (1968) High resolution techniques of peptide mapping. Separation of bovine carotid actin peptides on cellulose thin layers and of the corresponding dansyl-peptides on polyamide thin layers. *J Chromatogr* **38**: 408–411.

Goffin, C., and Ghuysen, J.-M. (1998) Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. *Microbiol Mol Biol Rev* **62**: 1079–1093.

Hilbert, F., del Portillo, F.G., and Groisman, E.A. (1999) A periplasmic D-alanyl-D-alanine dipeptidase in the gram-negative bacterium *Salmonella enterica*. *J Bacteriol* **181**: 2158–2165.

Holtje, J.V. (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiol Mol Biol Rev* **62**: 181–203.

Kato, Y., Asano, Y., Nakazawa, A., and Kondo, K. (1989) First stereoselective synthesis of D-amino acid N-alkyl amides catalysed by D-aminopeptidase. *Tetrahedron* **45**: 5743–5754.

Kawarabayasi, Y., Sawada, M., Horikawa, H., Haikawa, Y., Hino, Y., Yamamoto, S., *et al*. (1998) Complete sequence and gene organization of the genome of a hyper-thermophilic archaebacterium, *Pyrococcus horikoshii* OT3. *DNA Res* **5**: 55–76.

Kawarabayasi, Y., Hino, Y., Horikawa, H., Yamazaki, S., Haikawa, Y., Jin-no, K., *et al*. (1999) Complete genome sequence of an aerobic hyper-thermophilic crenarchaeon, *Aeropyrum pernix* K1. *DNA Res* **6**: 83–101.

Komeda, H., and Asano, Y. (2000) Gene cloning, nucleotide sequencing, and purification and characterization of the D-stereospecific amino-acid amidase from *Ochrobactrum anthropi* SV3. *Eur J Biochem* **267**: 2028–2035.

Kuan, G., Dassa, E., Saurin, W., Hofnung, M., and Saier, M.H., Jr (1995) Phylogenetic analyses of the ATP-binding constituents of bacterial extracytoplasmic receptor-dependent ABC-type nutrient uptake permeases. *Res Microbiol* **146**: 271–278.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., *et al*. (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* **390**: 249–256.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Ledent, P., Duez, C., Vanhove, M., Lejeune, A., Fonze, E., Charlier, P., *et al*. (1997) Unexpected influence of a C-terminal-fused His-tag on the processing of an enzyme and on the kinetic and folding parameters. *FEBS Lett* **413**: 194–196.

Lessard, I.A., and Walsh, C.T. (1999a) Mutational analysis of active-site residues of the enterococcal D-ala-D-Ala dipeptidase VanX and comparison with *Escherichia coli* D-ala-D-Ala ligase and D-ala-D-Ala carboxypeptidase VanY. *Chem Biol* **6**: 177–187.

Lessard, I.A., and Walsh, C.T. (1999b) Van X, a bacterial D-alanyl-D-alanine dipeptidase: resistance, immunity, or survival function? *Proc Natl Acad Sci USA* **96**: 11028–11032.

Mathiopoulos, C., Mueller, J.P., Slack, F.J., Murphy, C.G., Patankar, S., Bukusoglu, G., *et al*. (1991) A *Bacillus subtilis* dipeptide transport system expressed early during sporulation. *Mol Microbiol* **5**: 1903–1913.

Mitani, T., Heinze, J.E., and Freese, E. (1977) Induction of sporulation in *Bacillus subtilis* by decoyinine or hadacidin. *Biochem Biophys Res Commun* **77**: 1118–1125.

Park, K., and Reardon, K. (1996) Medium optimization for recombinant protein production by *Bacillus subtilis*. *Biotechnol Lett* **18**: 737–740.

Redenbach, M., Kieser, H.M., Denapaite, D., Eichner, A., Cullum, J., Kinashi, H., *et al*. (1996) A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. *Mol Microbiol* **21**: 77–96.

Reynolds, P.E., Depardieu, F., Dutka-Malen, S., Arthur, M., and Courvalin, P. (1994) Glycopeptide resistance mediated by enterococcal transposon Tn*1546* requires production of VanX for hydrolysis of D-alanyl-D-alanine. *Mol Microbiol* **13**: 1065–1070.

Robertson, J.B., Gocht, M., Marahiel, M.A., and Zuber, P. (1989) AbrB, a regulator of gene expression in *Bacillus*, interacts with the transcription initiation regions of a sporulation gene and an antibiotic biosynthesis gene. *Proc Natl Acad Sci USA* **86**: 8457–8461.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Saurin, W., Hofnung, M., and Dassa, E. (1999) Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. *J Mol Evol* **48**: 22–41.

Serror, P., and Sonenshein, A.L. (1996) Interaction of CodY, a novel *Bacillus subtilis* DNA-binding protein, with the dpp promoter region. *Mol Microbiol* **20**: 843–852.

Slack, F.J., Mueller, J.P., Strauch, M.A., Mathiopoulos, C., and Sonenshein, A.L. (1991) Transcriptional regulation of a *Bacillus subtilis* dipeptide transport operon. *Mol Microbiol* **5**: 1915–1925.

Slack, F.J., Mueller, J.P., and Sonenshein, A.L. (1993) Mutations that relieve nutritional repression of the *Bacillus subtilis* dipeptide permease operon. *J Bacteriol* **175**: 4605–4614.

Slack, F.J., Serror, P., Joyce, E., and Sonenshein, A.L. (1995) A gene required for nutritional repression of the *Bacillus subtilis* dipeptide permease operon. *Mol Microbiol* **15**: 689–702.

Teorell, T., and Stenhagen, E. (1938) Ein Universalpuffer für den pH-Bereich 2.0 bis 12.0. *Biochem Z* **299**: 416–419.

White, O., Eisen, J.A., Heidelberg, J.F., Hickey, E.K., Peterson, J.D., Dodson, R.J., *et al*. (1999) Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1. *Science* **286**: 1571–1577.