Site-directed mutagenesis of the hydrogenase signal peptide consensus box prevents export of a β -lactamase fusion protein

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A secretion vector, pVN1, expressing the [NiFe] hydrogenase signal peptide of *Desulfovibrio vulgaris* Hildenborough fused to β -lactamase from *Escherichia coli* was constructed in order to study the unusual characteristics of hydrogenase signal peptides, which share a strictly conserved sequence, the consensus box: R-R-X-F-X-K. Although the hydrogenase signal peptide- β -lactamase fusion protein was processed much more slowly than the fusion of β -lactamase with its own signal peptide, the system mimicked several features expected for hydrogenase biosynthesis in *E. coli*, including increased export under anaerobic conditions. Site-directed mutagenesis of R(-28), the first arginine residue of the consensus box, to a glutamate completely inhibited export and processing of the fusion protein. The same mutation of R(-33), located outside the consensus box, had almost no effect. The data indicate a specific role for the consensus box sequence in the export mechanism for hydrogenase.

Introduction

Proteins to be exported from the cell cytoplasm are synthesized as precursors containing an NH₂-terminal extension of 15–30 amino acid residues. This extension, the signal peptide, is the main determinant for protein localization to the periplasm. Following translocation across the cytoplasmic membrane, this signal peptide is cleaved by a specific membrane-bound signal peptidase, to yield the mature protein. Sequence comparison of signal peptides has shown that there is no strict sequence conservation among these peptides (Gierasch, 1989; Von Heijne, 1985; Watson, 1984). However, three different domains can be assigned to a classical signal peptide. These are: (i) an NH₂-terminal positively charged region, (ii) a central hydrophobic region of 8–10 amino acid residues, and (iii) a COOH-terminal segment which

includes the signal peptidase cleavage site (Schatz and Beckwith, 1990).

In the last seven years, the cloning and sequencing of structural genes for hydrogenases has revealed the presence of unusual signal peptides (Fig. 1). Hydrogenase, a bacterial enzyme, which catalyses the reversible oxidation of molecular hydrogen, is often located in the periplasmic space (Nivière *et al.*, 1991; Van der Westen *et al.*, 1978) or found associated with the membrane (Rhode *et al.*, 1990). Periplasmic hydrogenases consist of a large (45–65 kDa) and a small (9–30 kDa) subunit, encoded by a single operon. These enzymes always contain iron-sulphur clusters as prosthetic groups, and some also contain nickel. On the basis of the prosthetic group at the active site of the enzyme, three classes of hydrogenases have been described: the [Fe], [NiFe] and [NiFeSe] hydrogenases (Fauque *et al.*, 1988). The [NiFe] and [NiFeSe] hydrogenases have homologous primary sequences. However, the [Fe] hydrogenases do not share primary sequence homology with these two nickel-containing enzymes (Voordouw, 1990).

An NH₂-terminal signal peptide is only present in the small subunit (pro- β) of the three classes of hydrogenases (Fig. 1). The large (α) subunit does not have an NH₂-terminal signal peptide and no evidence for an internal signal sequence has so far been obtained. The pro- β signal peptides are exceptionally long (32–50 residues) and have a complex NH₂-terminal domain with positive

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Abbreviations: PADAC, 7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethylaminophenylazo)pyridinium methyl]-3-cephem-4 carboxylic acid.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M97299.

Sulphate-reducing bacteria

[illegible]

Nitrogen-fixing bacteria

Bj [NiFe]	-	++	++	+		+	-
	MGAATETFYSVIRRQGIT	RR	S	F	H	K	FCSLTATSLGLGPLAASRIANA LET
Av [NiFe]	+	-	++	++	+		+
	MSRLETFYDVMRROGIT	RR	S	F	L	K	YCSLTAAALGLGPAFAPRIAHA MET

Photosynthetic bacteria

Rc	[NiFe]	-	-	-	++	++	+	+		+	-	-
		MMSDIETFYDVMRRQGIT	RR	S	F	M	K	SVRSPQHVLGLGPSFVPKIGEA	MET			
Rq	[NiFe]	-	-	++	++	++	+					-
		METFYEVMRRGIS	RR	S	F	L	K	YCSLTATSLGLAPSFPQIAHA	MET			

Enterobacteria

Ec [NiFe]   --   ++   ++   +   +   -
MNNEETFYQAMRRQGVT RR S F L K YCSLAATSLGLGAGMAPKIAWA LEN

Consensus box :   ++   +
                                 RR   F   K

Fig. 1. Signal peptide sequences determined for small subunits of [Fe], [NiFe] and [NiFeSe] hydrogenases from different micro-organisms. The sequences have been aligned relative to the consensus sequence RRXFXXK. Positively (+; R, K) and negatively (-; E, D) charged amino acid residues are indicated. The following are the full names of the micro-organisms and references to the sequences: DvH [Fe] is *D. vulgaris* Hildenborough (Voordouw & Brenner, 1985); DvMo [Fe] is *D. vulgaris* oxamicus Monticello (Voordouw *et al.*, 1989*b*); Db [NiFeSe] is *D. baculatus* (Voordouw *et al.*, 1989*a*); DvH [NiFe] is *D. vulgaris* Hildenborough (this study); DvM [NiFe] is *D. vulgaris* Miyazaki (Deckers *et al.*, 1990); Dg [NiFe] is *D. gigas* (Voordouw *et al.*, 1989*a*); Df [NiFe] is *D. fructosovorans* (Rousset *et al.*, 1990); Bj [NiFe] is *Bradyrhizobium japonicum* (Sayavedra-Soto *et al.*, 1988); Av [NiFe] is *Azotobacter vinelandii* (Menon *et al.*, 1990*a*); Rc [NiFe] is *Rhodobacter capsulatus* (Leclerc *et al.*, 1988); Rg [NiFe] is *Rhodocyclus gelatinosus* (Uffen *et al.*, 1990); Ec [NiFe] is *Escherichia coli* (Menon *et al.*, 1990*b*).

and negative charges. Fig. 1 shows the sequence alignment of most of the known hydrogenase pro- β signal peptides. These signal peptides, from very different bacterial species, share a conserved sequence, the consensus box: RRFXFK. Such a strictly conserved sequence has never before been identified among signal peptides and its presence suggests that the hydrogenase secretion pathway is similarly conserved. This pathway has been studied in some detail (Menon *et al.*, 1991; Van Dongen *et al.*, 1988; Voordouw *et al.*, 1987; Voordouw, 1990). Its most notable feature is that export of both

subunits appears cooperative: e.g. export and processing of the small subunit of *Escherichia coli* [NiFe] hydrogenase-I expressed from its cloned gene in *E. coli* is only accomplished when the large subunit gene and other genes of the operon are also expressed (Menon *et al.*, 1991). In the case of expression of genes for [Fe] hydrogenase from *Desulfovibrio vulgaris* Hildenborough in *E. coli* coexpression of both genes for the small β and large α subunit led to export of only a small fraction of $\alpha\beta$ dimer (Van Dongen *et al.*, 1988). However, no export of either subunit was observed when only a single gene was

expressed. The cooperative nature of the hydrogenase assembly process precludes a study of the unique properties of the signal peptide by monitoring export and activation of the hydrogenase system. The specific properties of the hydrogenase signal peptide can also be studied by fusing a hydrogenase signal peptide to a simpler, exportable protein such as β -lactamase, that does not require subunit or metal ion cluster assembly for activation.

Methods

Biochemical reagents. All enzymes were obtained from Pharmacia. Radioactively labelled [α - ^{35} S]dATP (400 Ci mmol $^{-1}$, 10 mCi ml $^{-1}$; 1 Ci = 37 GBq) for dideoxy-sequencing was from Amersham. Tran ^{35} S-label, containing 70% L-[^{35} S]methionine and 15% L-[^{35} S]cysteine (1026 Ci mmol $^{-1}$, 10 mCi ml $^{-1}$), used for pulse-chase labelling, was obtained from ICN Biochemicals. Deoxyoligonucleotides were purchased from the Regional DNA Synthesis Laboratory of the University of Calgary. PADAC was from Calbiochem. Prestained SDS-PAGE low molecular mass range standards were from Bio-Rad. Nitrocellulose membrane was from Schleicher and Schuell. Reagent-grade chemicals were obtained from either Fisher, Sigma or Pharmacia.

Strains, vectors and media. The bacterial strains, plasmids and cloning vectors used in this work are described in Table 1. Aerobic cultures were grown in TY medium containing 10 g bactotryptone, 5 g yeast extract and 5 g NaCl per litre of water at pH 7.4 (aerobic TY medium). Anaerobic cultures were grown in TY medium supplemented with 30 mM-formate, 0.4% (w/v) glucose, 2 μ M-sodium selenite and 2 μ M-ammonium molybdate (anaerobic TY medium). Aerobic glucose minimal medium contained 6.25 g Na₂HPO₄, 0.75 g KH₂PO₄, 2 g NaCl, 0.2 g MgSO₄·7H₂O, 1 g (NH₄)₂SO₄, 10 mg FeSO₄·7H₂O, 10 mg Na₂MoO₄·2H₂O, 0.16 mg NaSeO₃ and 4 g glucose per litre of deionized water at pH 7.4, as described by Lee *et al.* (1990). Anaerobic glucose minimal medium contained 10 g glucose per litre and also 30 mM-formate.

Plasmid construction. DNA manipulations were as described by Sambrook *et al.* (1989). Plasmids were transformed into *E. coli* TG108 and transformants were selected by their resistance to kanamycin (50 μ g ml $^{-1}$) or ampicillin (100 μ g ml $^{-1}$). Plasmid pKUL6 (Fig. 2) was constructed as follows. A 1.38 kb *Dra*I–*Nde*I fragment containing the plasmid replication origin was obtained from pUC19. End-repair of this fragment and ligation with a 1.2 kb kanamycin resistance cartridge (*Bam*HI fragment, end-repaired) from pUC-4K gave pKUL6. Ligation of *Kpn*I- and *Sac*I-digested pKUL6 with the 2 kb *Kpn*I–*Sac*I fragment from pWB (Wong, 1989), expressing a levansucrase– β -lactamase fusion, generated pKUL7 (Fig. 2; Fig. 3*b*, iv). For construction of pVN1 (Fig. 2), plasmid DV1, containing two *Cl*aI sites, was partially digested with *Cl*aI (note: only the *Cl*aI site targeted for modification is shown in Fig. 2), end-repaired with Klenow polymerase and dNTPs and *Bam*HI linkers (5'pCCGGATCCGG) ligated into the blunt-ended site. Plasmid pGV21, with a single *Bam*HI site, was digested to completion with *Nae*I, which leaves the desired ~330 nucleotide (nt) fragment connected to the 3.0 kb BLUESCRIPT vector (Stratagene), and religated in the presence of *Bam*HI linkers to give pGV32 with two *Bam*HI sites flanking the desired ~330 nt *Bam*HI fragment. Plasmid pKUL7, obtained by *Bam*HI digestion and religation of pKUL6 (Fig. 2), was digested with *Bam*HI and ligated to the ~330 nt *Bam*HI fragment of pGV32 to give pVN1 (Fig. 2; Fig. 3*a*). Plasmid pVN1, expressing an in-frame fusion of the [NiFe] hydrogenase signal sequence and mature β -lactamase from the *lac* promoter, was verified by restriction digestion

Table 1. Bacterial strains and DNA vectors used in this study

Strain/vector	Genotype, comments and reference
<i>E. coli</i> TG2	$\Delta(lac-pro) supE thi hsdM hsdR recA F'$ (<i>traD36 proAB⁺ lacZ</i> Δ M15I ^a) (from T. J. Gibson, MRC Centre, Cambridge, UK)
<i>E. coli</i> TG108	As <i>E. coli</i> TG2 but lacking F' (this study)
<i>E. coli</i> RZ1032	Hfr KL16 PO/45 [<i>lysA</i> (61–62)] <i>dut-1 ung-1 thi-1, relA1 Zbd-279::Tn10 supE44</i> (Kunkel <i>et al.</i> , 1987)
DV1	Contains the structural genes for the [NiFe] hydrogenase from <i>D. vulgaris</i> Hildenborough in BLUESCRIPT; Ap ^R (from A. E. Przybyla, University of Georgia, USA)
PGV21	Derived from DV1; Ap ^R (this study)
PGV32	Derived from pGV21; Ap ^R (this study)
pKUL6	Contains the levansucrase signal peptide gene fused in-frame to the gene of mature β -lactamase; Km ^R (this study)
pKUL7	Derived from pKUL6; contains the gene for mature β -lactamase without signal peptide; Km ^R (this study)
pVN0	Derived from pKUL7; contains the wild-type <i>bla</i> gene; Km ^R (this study)
pVN1	Derived from pKUL7; contains the [NiFe] hydrogenase signal peptide fused in-frame to the gene of the mature β -lactamase; Km ^R (this study)
pIP101	Contains the <i>lacZ</i> gene; Ap ^R (from B. Muller-Hill, University of Cologne, Germany)
pWB	(Wong, 1989)
pBR322, pUC19, M13mp18, pUC-4K	Purchased from Pharmacia (Sambrook <i>et al.</i> , 1989)

and dideoxynucleotide sequencing of M13 subclones (Sanger *et al.*, 1977). Plasmid pVN0, expressing β -lactamase with the native signal peptide (Fig. 3*b*, v) from tandemly arranged *lac* and *bla* promoters, was obtained by digestion of pVN1 with *Hind*III and *Sac*I and ligation with a 546 nt *Hind*III–*Sac*I fragment from pBR322.

Oligonucleotide-directed mutagenesis. The Kunkel method (Kunkel *et al.*, 1987) was used for mutagenesis. The 330 nt *Bam*HI fragment from pVN1 was cloned into the replicative form of M13mp18. The template, obtained from *E. coli* RZ1032, was extended from a hybridized mutagenic primer and ligated using Klenow polymerase, dNTPs, T4 DNA ligase and ATP. Mutagenic primer P49N, 5'GAAGTCGCGT-(A,T)(C,T)GCTGACGCC, changed the CGA codon for R(–28) at nt 223–225 (Fig. 3) into either GAA (E), GTA (V), ATA (I) or AAA (K). P50N, 5'GACGCCACGC(A,T)(C,T)CGCCAGACG, changed the CGG codon for R(–33) at nt 208–210 of the signal peptide into either GAG (E), GTG (V), AAG (K) or ATG (M). P51N, 5'CGGTCAGCT-CACGCGCC changed the GCG codon for A(–1) into GAG (E). Mutagenic primer P60N deleted nt 223–240, encoding the consensus residues RRDFLK (Fig. 3*b*, ii), while P59N deleted nt 161–238, encoding R(–49) to L(–24) (Fig. 3*b*, iii). After transfection of the extension-ligation mixtures into *E. coli* TG2, mutant M13 DNAs were identified by dideoxy-sequencing (Sanger *et al.*, 1977). The *Bam*HI insert of the replicative form of the M13 DNA carrying the verified mutation was then ligated to *Bam*HI digested pKUL7. The presence of the insert in the correct orientation was verified by double digestion with *Apa*I and *Sac*I (Figs 2 and 3).

Growth of bacteria. *E. coli* TG108 transformants were grown in 100 ml cultures at 37 °C in either aerobic or anaerobic TY medium

Fig. 2. Construction of pVN1, encoding a fusion of the *D. vulgaris* Hildenborough *[NiFe]* hydrogenase signal peptide (*spH*) to the mature β -lactamase (*bla*). Plasmid pKUL6 encodes a similar fusion of the levansucrase signal peptide, which is indicated by *spL* (see Fig. 3b, iv)

supplemented with 50 μ g kanamycin ml^{-1} and shaken at 250 r.p.m. in a New Brunswick G25 shaker for the determination of β -lactamase activity and Western blot analysis. For pulse-chase experiments, 5 ml cultures of transformants were grown at 37 °C in either aerobic or anaerobic glucose minimal medium supplemented with 400 μ g L-proline, 1 μ g of thiamin and 50 μ g kanamycin ml^{-1} . Anaerobic growth was in vials closed with rubber stoppers containing medium flushed with nitrogen. Samples of exponential-phase cultures were taken at an OD_{600} of 0.70–0.80.

Pulse-chase labelling. Exponential-phase cells (5 ml) were labelled for 2 min by adding 50 μ l Tran^{35}S -label (10 $\mu\text{Ci } \mu\text{l}^{-1}$). Chase was initiated by addition of 50 μ l minimal medium with unlabelled L-methionine (250 mM) and L-cysteine (60 mM). Samples (0.5 ml) were removed at the

indicated times (Fig. 4) and immediately injected into 0.5 ml ice-cold 10% (w/v) trichloroacetic acid. Precipitates were pelleted, washed with 1 ml ice-cold acetone and dried under vacuum. The dried pellets were resuspended in 50 μ l 10 mM-Tris/HCl, pH 8.0, 1% (w/v) SDS, 1 mM-EDTA, and solubilized by heating for 5 min in a boiling water bath. For immunoprecipitation, a 30 μ l portion of a solubilized sample was added to 1 ml Triton buffer (Ito *et al.*, 1981) together with 8 μ l purified polyclonal anti- β -lactamase antibodies (0.6 mg ml^{-1}). Purification was by immuno-affinity chromatography using β -lactamase immobilized on CNBr-activated Sepharose-4B (Harlow & Lane, 1988). Immunoprecipitates were pelleted with Protein A-Sepharose and washed as described by Plückthun & Knowles (1987). The resulting pellets were boiled for 5 min with 100 μ l SDS loading buffer [10% (w/v) SDS, 250 mM-Tris/HCl, pH 6.8, 50% (w/v) glycerol, 0.5% (w/v) bromo-

phenol blue, 5% (w/v) 2-mercaptoethanol] prior to loading on SDS-polyacrylamide gels. For cell fractionation, 0.5 ml of pulse-chase labelled cells were incubated on ice for 10 min before the fractionation procedure was started (see below). Periplasmic and spheroplast fractions were similarly precipitated with trichloroacetic acid, washed, dried, resuspended in 50 µl 10 mM-Tris/HCl, pH 8.0, 1% (w/v) SDS, 1 mM-EDTA and boiled for 5 min. The membrane fraction was directly resuspended in 50 µl 10 mM-Tris/HCl, pH 8.0, 1% (w/v) SDS, 1 mM-EDTA and boiled for 5 min. Immunoprecipitation and incubation with SDS loading buffer of these fractions were done as described for the whole cells.

Cell fractionation. Periplasmic, spheroplast and membrane fractions were prepared at 4°C. The pellet of exponential-phase cells was suspended in 300 µl 50 mM-Tris/HCl, pH 8.0, 10% (w/v) sucrose in a microfuge tube. Lysozyme (50 µl, 2 mg ml⁻¹) was then added. Following incubation for 5 min, 600 µl 50 mM-Tris/HCl, pH 8.0, 15 mM-EDTA and 10% (w/v) sucrose was added and incubation continued for 5 min. The microfuge tube was then centrifuged at 16000 *g* for 5 min. The supernatant was taken as the periplasmic fraction. The spheroplast pellet was lysed by resuspension in 1 ml 0.1% (w/v) Triton X-100 and incubation for 5 min to obtain the cytoplasmic/membrane fraction. Fractionation of *E. coli* TG108, transformed with pIP101, carrying the *lacZ* and *bla* genes for cytoplasmic β -galactosidase and periplasmic β -lactamase, indicated 98% of the β -galactosidase activity in the spheroplast pellet, and 95% of the β -lactamase activity in the periplasmic fraction. For membrane fraction preparation, the spheroplast pellet was resuspended in 1 ml 10 mM-Tris/HCl, pH 8.0, 1 mM-EDTA, 1 µg DNAase I ml⁻¹ and subjected to three cycles of freezing and thawing. The pellet from centrifugation at 150000 *g* for 2 h was resuspended in 1 ml 50 mM-Tris/HCl, pH 8.0, 0.5 M-NaCl and recentrifuged under the same conditions.

Enzyme activity measurements. In assays of β -lactamase activity PADAC was used as the substrate at a concentration corresponding to $A_{570} \sim 1$ in 100 mM-sodium phosphate buffer, pH 7.0. The activity was expressed in units per 10⁸ cells, where one unit is defined as the amount of enzyme hydrolysing 1 µmol PADAC min⁻¹ and an OD₆₀₀ of 1 was assumed to correspond to 8×10^8 cells ml⁻¹ (Sambrook *et al.*, 1989).

SDS-PAGE and Western blotting. SDS-PAGE was done on 12% (w/v) polyacrylamide running gels according to Laemmli (1970). Molecular masses of sample proteins were determined relative to prestained SDS-PAGE low-range molecular mass standards. For autoradiography, gels were shaken in 10% (v/v) glacial acetic acid, 20% (v/v) methanol for 30 min, then in 5% (v/v) glycerol, 50% (v/v) methanol for 30 min, dried and exposed to X-ray film for 2–3 d. For Western blotting analysis, 100 µl SDS loading buffer was added directly to 1 ml samples of exponential-phase aerobic or anaerobic TY cultures. Following boiling of the samples for 5 min, SDS-PAGE and electrophoretic transfer to nitrocellulose (Towbin *et al.*, 1979), the nitrocellulose filters were processed as reported elsewhere (Pollock *et al.*, 1989) except that purified polyclonal anti- β -lactamase serum was used as the primary antibody at a 1:500 dilution. An alkaline-phosphatase-conjugated goat anti-rabbit IgG was used as the secondary antibody and the blot was immunostained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Results

Construction of pVN1

The relevant part of the nucleotide sequence of pVN1, constructed as outlined in Fig. 2, is shown in Fig. 3(a).

The construction procedure generated codons for five additional amino acid residues: PGSGI (Fig. 3b, i: +6 to +10). The amino acid sequence of *D. vulgaris* [NiFe] hydrogenase ends at R(+5), while that of β -lactamase starts at P(+11), which is residue (+2) of mature β -lactamase (Fig. 3b, v). The construction conserves the hydrogenase signal peptide cleavage site. A ribosome-binding site (RBS), AAGGAGG, is present 6 nucleotides upstream from the ATG initiation codon of the hydrogenase signal peptide. Expression of the *bla* gene fusion is under control of the *lac* promoter (Fig. 2). Addition of IPTG is not necessary for maximal expression in *E. coli* TG108, since the *lacI*^q gene encoding *lac* repressor is deleted from this strain (Table 1).

Pulse-chase labelling analysis of fusion protein processing and export

Because *E. coli* specifically expresses hydrogenase activity under anaerobic conditions, experiments were carried out under both aerobic and anaerobic conditions. The results of several pulse-chase labelling experiments are shown in Fig. 4. The purified polyclonal anti- β -lactamase serum specifically immunoprecipitated five forms of β -lactamase, **a–e**, from *E. coli* TG108(pVN1). Form **c**, molecular mass 29.0 kDa, only appeared after a period of chase, was the only form present in the periplasmic fraction (Fig. 4b) and represents, therefore, the processed and exported form of β -lactamase. Form **b** (molecular mass 34.5 kDa) disappeared during pulse-chase labelling, in parallel with the appearance of the periplasmic form **c** (Fig. 4a, c). Its cytoplasmic location (Fig. 4b) and molecular mass indicate it to represent the fusion protein as translated in Fig. 3(a). Additional evidence that form **b** is converted into **c** by proteolytic processing at the site indicated in Fig. 3 came from mutagenesis of the codon for A(–1). The mutant expressed the 34.5 kDa pre- β -lactamase (form **b**) but the A(–1)E mutation prevented processing to form **c** (not shown). Form **a** (molecular mass 38.0 kDa), which disappeared with a similar rate as **b** (Fig. 4a, c), could result from translation initiation at a TTG codon (nt 85). The sequence AGGTG (nt 70) is complementary to the 3' end of *E. coli* 16 S rRNA and is spaced the optimal distance of 10 nt from the TTG codon, which can serve as an initiation codon in *E. coli*. Alternatively, translation may initiate at the ATG codon at nt 88. The resulting polypeptide has a calculated molecular mass of 37.0 kDa. Forms **d** and **e** (28.0 and 26.0 kDa) remained relatively constant during pulse-chase labelling (Fig. 4a) and were mainly located in the cytoplasm (Fig. 4b). They were the only forms observed in experiments with *E. coli* TG108(pKUL7), which lacks a signal peptide, and must

Fig. 3. (a) Nucleotide sequence of a 351 nt portion of plasmid pVN1, encoding the NH₂ terminus of the [NiFe] hydrogenase signal peptide- β -lactamase fusion protein, which is translated using the single-letter amino acid code. The hydrogenase signal peptidase cleavage site is indicated (↓), as are the sites for restriction endonucleases *Bam*HI, *Apa*I and *Eco*RI. A putative ribosome-binding site (RBS) is also indicated. (b) (i) Mutations generated in the hydrogenase signal sequence at R(-33), R(-28) and A(-1). (ii), (iii) Deletion mutations of the hydrogenase signal sequence. (iv) *Bacillus subtilis* levansucrase signal sequence- β -lactamase fusion expressed by plasmid pKUL6 (Fig. 2). (v) Native signal sequence- β -lactamase expressed by pVN0.

result from translation initiation at positions downstream from nt 157.

Pulse-chase labelling of *E. coli* TG108(pVN1) (Fig. 4a) indicated that the periplasmic β -lactamase, form c, appeared at 10–20 min after the start of the chase. Repeating the same experiment with *E. coli* TG108(pVN0), encoding β -lactamase with its native signal peptide (Fig. 3b, v), showed more rapid and efficient processing of pre-Bla (molecular mass 31.5 kDa) to Bla (molecular mass 28.9 kDa). The cytoplasmic form e, molecular mass 26 kDa, was also

observed but in lower concentration (Fig. 4d). Comparing the results of Fig. 4(a) and 4(d) indicates that the form b to c conversion in *E. coli* TG108(pVN1) is clearly slower and less efficient than the conversion of pre-Bla to Bla in *E. coli* TG108(pVN0). In *E. coli*, hydrogenase is specifically expressed under anaerobic conditions in the presence of glucose or formate (Sawers *et al.*, 1985). Interestingly, processing of the fusion protein occurred more rapidly under these conditions (Fig. 4c: form c appears after 2–5 min), compared to aerobic conditions (Fig. 4a).

Fig. 4. Pulse-chase labelling of β -lactamase proteins. Following a 2 min pulse with [^{35} S]methionine/cysteine the cells were chased with non-radioactive methionine/cysteine for the indicated times. (a) *E. coli* TG108(pVN1) grown in aerobic glucose minimal medium. (b) Fractionation of whole cells (W) of *E. coli* TG108(pVN1) grown in aerobic glucose minimal medium after a 20 min chase into a periplasmic (P), spheroplast (Sph) and membrane (M) fraction. The spheroplast fraction contains both membrane and cytoplasm fractions. (c) *E. coli* TG108(pVN1) grown in anaerobic glucose minimal medium. (d) *E. coli* TG108(pVN0) grown in aerobic glucose minimal medium. Bands **b** and **c** are the precursor (34.5 kDa) and processed (29 kDa) forms of the β -lactamase fusion protein. Bands **a**, **d** and **e** of 38, 28 and 26 kDa, respectively, also react specifically with the serum and are discussed in the text. The native precursor (pre-Bla, 31.5 kDa) and its processed form (Bla, 29 kDa) are indicated.

Site-directed mutagenesis of the consensus box residue R(-28)

To identify which parts of the hydrogenase signal sequence are important for export of the fusion protein some initial deletion studies were carried out. Pulse-chase experiments with a variant of pVN1 in which the consensus box sequence was deleted (Fig. 3*b*, ii) indicated absence of processing of the precursor form **b** (molecular mass 33.9 kDa) to **c** (not shown). Deletion of most of the NH₂-terminal charged region (Fig. 3*b*, iii) to a signal peptide of 24 residues, similar in length to the native signal (23 residues) also inhibited processing of form **b** (molecular mass 31.6 kDa). These results indicate that the presence of the consensus box sequence is important for fusion protein processing, but do not prove that the strictly conserved hydrogenase signal peptide consensus box RRXFXK (Fig. 1) is required. We therefore constructed four mutants which synthesized a fusion protein in which R(-28) was replaced by

either E, V, K or I. Rather than by pulse-chase experiments the effect of each mutation on the export of the β -lactamase fusion protein was determined by β -lactamase activity measurements of cell fractions, while processing was monitored by Western blot analysis of whole cells. The wild-type hydrogenase signal peptide directed 60% of the total β -lactamase activity to the periplasm under aerobic and 86% under anaerobic conditions. The absolute value of the periplasmic β -lactamase activity increased threefold, from 0.18 to 0.55 units per 10⁸ cells, under anaerobic conditions (Table 2). Western blotting experiments with wild-type signal peptide indicated more complete processing of the precursor form **b** to **c** under anaerobic conditions (Fig. 5, lanes 1 and 2): form **b** cannot be detected in Western blot experiments with wild-type signal peptide under these conditions. These results agree with the observation of more rapid fusion protein processing in pulse-chase experiments under anaerobic conditions. The classical native or levansucrase signal peptides (Fig. 3*b*, iv and v)

Fig. 5. Effect of mutation of signal peptide residue R(-28) on the processing of the β -lactamase fusion protein. Whole cells of *E. coli* TG108 transformed with pVN1 or a mutant form of this plasmid, grown in aerobic (odd-numbered lanes) or anaerobic (even-numbered lanes) TY medium were analysed by SDS-PAGE and Western blotting, using a polyclonal β -lactamase antiserum. Lanes 1 and 2, wild-type; lanes 3 and 4, R(-28)E; lanes 5 and 6, R(-28)V; lanes 7 and 8, R(-28)I; lanes 9 and 10, R(-28)K. Lane 11 represents untransformed *E. coli* TG108. The various forms a-e of β -lactamase are explained in the legend to Fig. 4. Pre β -lact and Mat β -lact represent the precursor and the mature forms b and c of β -lactamase respectively. Bands f and g react non-specifically with the serum and are also found in whole cells of *E. coli* TG108 without plasmid (lane 11).

directed 95% of the β -lactamase activity to the periplasm under both aerobic and anaerobic conditions. This higher fraction is again consistent with the more rapid processing observed in Fig. 4(d).

The β -lactamase activities measured for the R(-28) mutants are summarized in Table 2. The R(-28)E mutation strongly affected the export of β -lactamase to the periplasm. Compared to the wild-type signal peptide, we observed an 84–93% decrease of periplasmic β -lactamase activity. The values obtained for this mutant were actually very similar to those for *E. coli* TG108(pKUL7), where the signal peptide has been deleted. The other mutations, R(-28)V, R(-28)I and R(-28)K, had less strong effects (Table 2).

Western blotting indicated that the R(-28)E mutation (Fig. 5: lanes 3, 4) completely prevented processing of pre- β -lactamase to form c. No differences could be noted between aerobic (lane 3), and anaerobic (lane 4) growth conditions, in agreement with the results of the activity measurements. The effects of the other mutations are less pronounced (lanes 5–10). Under anaerobic conditions, pre- β -lactamase (form b) was only partially processed for these mutations (lanes 6, 8 and 10) compared to the wild-type control (lane 2).

Site-directed mutagenesis of R(-33)

To verify the specificity of the effects of the R(-28) mutation, we mutated another arginine residue, located outside the consensus box. R(-33) was considered to be a good control mutation, since, like R(-28), it is the first of an arginine doublet and the sequence on its COOH-terminal side (RRXXXXR) is similar to that on the COOH-terminal side of R(-28), which is RRXXXXK (Fig. 3b). Compared to the wild-type signal peptide, mutations R(-33)E, R(-33)M and R(-33)V decreased the

Table 2. Effects of hydrogenase signal peptide mutations on β -lactamase export

The codons for R(-28) and R(-33) were changed by site-directed mutagenesis. Partitioning of the β -lactamase activity expressed by the resulting derivatives of pVN1, transformed into *E. coli* TG108, was determined following growth in either aerobic or anaerobic TY medium.

Mutation	Growth condition	β -Lactamase activity*		Periplasmic activity ratio†
		Cytoplasmic‡	Periplasmic	
Wild-type	Anaerobic	0.09	0.55	100
	Aerobic	0.12	0.18	100
R(-28)E	Anaerobic	0.16	0.04	7
	Aerobic	0.12	0.03	16
R(-28)V	Anaerobic	0.12	0.24	43
	Aerobic	0.17	0.13	72
R(-28)I	Anaerobic	0.10	0.22	40
	Aerobic	0.12	0.09	50
R(-28)K	Anaerobic	0.08	0.22	40
	Aerobic	0.09	0.13	72
R(-33)E	Anaerobic	0.04	0.44	73
	Aerobic	0.13	0.14	77
R(-33)V	Anaerobic	0.10	0.50	91
	Aerobic	0.08	0.14	77
R(-33)M	Anaerobic	0.06	0.41	74
	Aerobic	0.06	0.12	66
R(-33)K	Anaerobic	0.15	0.57	104
	Aerobic	0.17	0.21	116
Signal peptide deleted§	Anaerobic	0.18	0.02	4
	Aerobic	0.14	0.02	11

* β -Lactamase activity is expressed in mmol PADAC min⁻¹ (10⁸ cells)⁻¹.

† This includes both cytoplasmic and membrane-bound activities.

‡ Ratio [(periplasmic activity)/(periplasmic activity of wild-type)] × 100.

§ Data for *E. coli* TG108(pKUL7).

export of β -lactamase activity by 10–34%. The mutation R(-33)K had no effect. Analysis by Western blotting of whole-cell fractions did not reveal significantly different processing of mutant signal sequences relative to the

wild-type signal sequence, under either aerobic or anaerobic conditions (data not shown). Thus, while some effects of mutating R(-33) were observed, they were small compared to those caused by mutating the consensus box residue R(-28).

Discussion

One of the most striking features in all periplasmic hydrogenase small subunit signal peptides characterized to date is the presence of the consensus box RRXFXK. This element is separated by a conserved number of residues from the signal peptide cleavage site, 21 residues in the [Fe] and 22 residues in the [NiFe] and [NiFeSe] hydrogenases (Fig. 1), and precedes the hydrophobic core of the signal peptide. The length variation of hydrogenase signal peptides (32–50 amino acids) is thus principally caused by a variable NH₂-terminal part preceding the positively charged consensus box. For [Fe] and [NiFeSe] hydrogenase signal peptides (32–34 residues), no extra charges are present in this region (4–7 residues). However, in [NiFe] hydrogenase signal peptides (41–50 residues), this region (14–22 residues) contains several positively and negatively charged amino acid side chains. Interestingly, these signal peptides exhibit some structural homology with the transit peptides which target proteins to intermembrane spaces of eukaryotic organelles such as the yeast mitochondrial cytochrome *c*₁. These mitochondrial signal peptides also have a long charged NH₂-terminal portion preceding their hydrophobic core (Pugsley, 1989). Whereas periplasmic [Fe] and [NiFeSe] hydrogenases seem to be restricted to sulphate-reducing bacteria of the genus *Desulfovibrio* (Voordouw *et al.*, 1990), the [NiFe] hydrogenase is widely distributed among micro-organisms, including sulphate-reducing bacteria, nitrogen-fixing bacteria, photosynthetic bacteria and enterobacteria (Fig. 1).

We have shown here that the hydrogenase signal peptide functions also in the export of β -lactamase, although less well than either the native β -lactamase or the *B. subtilis* levansucrase signal peptides (Fig. 3*b*, iv and v). Despite this limited efficiency the fusion protein system shows two features that indicate it to mimic hydrogenase export correctly: (i) export and processing of the fusion protein is dependent on the consensus box sequence, which cannot be deleted or mutagenized, and (ii) export and processing in *E. coli* appears more efficient under anaerobic conditions.

With respect to (i) we have shown that mutation of R(-28), the first residue of the consensus box, strongly inhibits export and processing of the fusion protein (Table 2, Fig. 5). These results are quite unusual for a

signal peptide. Indeed, several studies on the positively charged NH₂-terminal part of *E. coli* signal peptides have shown that the number of positive charges is unimportant as long as the global charge of the NH₂-terminal region remains positive (Kadonaga *et al.*, 1985; Lehnhardt *et al.*, 1988; Puziss *et al.*, 1989). In some cases, even a shift from a global positive to a global neutral charge does not significantly affect the efficiency of export (Vlasuk *et al.*, 1983). Mutations known to generate strong defects in protein export are mostly localized in the hydrophobic core of the signal peptide, changing a hydrophobic residue to a polar one (Gennity *et al.*, 1990). The mutation R(-28)E does not reverse the charge of the NH₂-terminal part of the signal peptide: its global charge remains still positive. Also, three other glutamate residues, E(-41), E(-38) and E(-37), are already present in this region. The strong inhibition of export and processing therefore indicates that the consensus box residue R(-28) plays a crucial role in the export process, especially in view of the fact that mutation of R(-33) has little effect.

With respect to (ii) it is important to establish whether fusion protein expression (the sum of transcription and translation, as well as mRNA and protein degradation) is altered in shifting from aerobic to anaerobic growth conditions. The data in Fig. 5, lanes 3 and 4, indicate that expression of the R(-28)E mutant form of the fusion protein, which is not processed, is unaltered. Since all other constructs for which data are shown in Fig. 5 differ only in the codon for R(-28), and since large variations in either transcription or translation rates due to these minor template changes appear unlikely, it is reasonable to assume that the expression levels in all of the samples analysed in Fig. 5 were similar and that the observed shifts were caused by changes in fusion protein processing rates. An increased processing rate under anaerobic conditions is evident from pulse-chase experiments. Although the two- to fivefold rate increase observed is modest, it accounts for the threefold increased level of wild-type periplasmic β -lactamase activity observed under anaerobic conditions. (Table 2). The question is, why do anaerobic conditions increase the rate of fusion protein processing in *E. coli*? Genetic studies have shown that many accessory genes are involved in the synthesis of active hydrogenases in *E. coli* in addition to the structural genes for these enzymes (e.g. Bohm *et al.*, 1990; Lutz *et al.*, 1991; Menon *et al.*, 1991). Their specific roles are largely unknown. We speculate that one of the accessory genes encodes a protein that specifically binds to the hydrogenase signal peptide consensus box and facilitates the export and processing of hydrogenase. In *E. coli*, expression of hydrogenase structural and accessory genes occurs only under anaerobic conditions (Sawers *et al.*, 1985; Sawers & Boxer, 1986), and we

postulate that the changed molecular composition of the *E. coli* cell under these conditions is responsible for the increased rate of fusion protein processing.

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References

- BÖHM, R., SAUTER, M. & BÖCK, A. (1990). Nucleotide sequence and expression of an operon in *Escherichia coli* coding for formate hydrogenylase components. *Molecular Microbiology* **4**, 231–243.
- DECKERS, H. M., WILSON, F. R. & VOORDOUW, G. (1990). Cloning and sequencing of a [NiFe] hydrogenase operon from *Desulfovibrio vulgaris* Miyazaki F. *Journal of General Microbiology* **136**, 2021–2028.
- FAUQUE, G., PECK, H. D., JR, MOURA, J. J. G., HUYNH, B. H., BERLIER, Y., DERVARTANIAN, D. V., TEIXEIRA, M., PRZYBYLA, A. E., LESPINAT, P. A., MOURA, I. & LE GALL, J. (1988). The three classes of hydrogenases from sulfate reducing bacteria of the genus *Desulfovibrio*. *FEMS Microbiology Reviews* **54**, 299–344.
- GENNITY J., GOLDSTEIN, J. & INOUE, M. (1990). Signal peptide mutants of *Escherichia coli*. *Journal of Bioenergetics and Biomembranes* **22**, 233–269.
- GIERASCH, L. M. (1989). Signal sequences. *Biochemistry* **28**, 923–930.
- HARLOW, E. & LANE, D. (1988). *Antibodies: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- ITO, K., BASSFORD, P. J., JR & BECKWITH, J. (1981). Protein localization in *E. coli*: is there a common step in the secretion of periplasmic and outer-membrane proteins? *Cell* **24**, 707–717.
- KADONAGA, J. T., PLÜCKTHUN, A. & KNOWLES, J. R. (1985). Signal sequence mutants of β -lactamase. *Journal of Biological Chemistry* **260**, 16192–16199.
- KUNKEL, T. A., ROBERTS, J. D., & ZAKOUR, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods in Enzymology* **154**, 367–382.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LECLERC, M., COLBEAU, A., CAUVIN, B. & VIGNAIS, P. M. (1988). Cloning and sequencing of the genes encoding the large and the small subunits of the H_2 uptake hydrogenase (*hup*) of *Rhodobacter capsulatus*. *Molecular and General Genetics* **214**, 97–108.
- LEE, J.-H., WENDT, J. C. & SHANMUGAM, K. T. (1990). Identification of a new gene, *molR*, essential for utilization of molybdate in *Escherichia coli*. *Journal of Bacteriology* **172**, 2079–2087.
- LEHNHARDT, S., POLLITT, N. S., GOLDSTEIN, J. & INOUE, M. (1988). Modulation of the effects of mutations in the basic region of the *OmpA* signal peptide by the mature portion of the protein. *Journal of Biological Chemistry* **263**, 10300–10303.
- LUTZ, S., JACOBI, A., SCHLENSOG, V., BÖHM, R., SAWERS, G. & BÖCK, A. (1991). Molecular characterization of an operon (*hyp*) necessary for the activity of the three hydrogenase isoenzymes in *Escherichia coli*. *Molecular Microbiology* **5**, 123–135.
- MENON, A. L., STULTS, L. W., ROBSON, R. L. & MORTENSON, L. E. (1990a). Cloning, sequencing and characterization of the [NiFe] hydrogenase-encoding structural genes (*hoxK* and *hoxG*) from *Azotobacter vinelandii*. *Gene* **96**, 67–74.
- MENON, N. K., ROBBINS, J., PECK, H. D., JR, CHATELUS, C. Y., CHOI, E. S. & PRZYBYLA, A. E. (1990b). Cloning and sequencing of a putative *Escherichia coli* [NiFe] hydrogenase-1 operon containing six open reading frames. *Journal of Bacteriology* **172**, 1969–1977.
- MENON, N. K., ROBBINS, J., WENDT, J. C., SHANMUGAM, K. T. & PRZYBYLA, A. E. (1991). Mutational analysis and characterization of the *Escherichia coli* *hya* operon, which encodes [NiFe] hydrogenase 1. *Journal of Bacteriology* **173**, 4851–4861.
- NIVIÈRE, V., BERNADAC, A., FORGET, N., FERNÁNDEZ, V. M. & HATCHIKIAN, C. E. (1991). Localization of hydrogenase in *Desulfovibrio gigas* cells. *Archives of Microbiology* **155**, 579–586.
- PLÜCKTHUN, A. & KNOWLES, J. R. (1987). The consequences of stepwise deletions from the signal-processing site of β -lactamase. *Journal of Biological Chemistry* **262**, 3951–3957.
- POLLOCK, W. B. R., CHEMERIKA, P. J., FORREST, M. E., BEATTY, J. T. & VOORDOUW, G. (1989). Expression of the gene encoding cytochrome c_3 from *Desulfovibrio vulgaris* Hildenborough in *Escherichia coli*: export and processing of the apoprotein. *Journal of General Microbiology* **135**, 2319–2328.
- PUGSLEY, A. P. (1989). *Protein Targeting*. San Diego: Academic Press.
- PUZZI, J. W., FIKES, J. D. & BASSFORD, P. J., JR (1989). Analysis of mutational alterations in the hydrophilic segment of the maltose-binding protein signal peptide. *Journal of Bacteriology* **171**, 2303–2311.
- RHODE, M., FÜRSTENAU, U., MAYER, F., PRZYBYLA, A. E., PECK, H. D., JR, LE GALL, J., CHOI, E. S. & MENON, N. K. (1990). Localization of membrane-associated [NiFe] and [NiFeSe] hydrogenases of *Desulfovibrio vulgaris* using immunoelectron microscopic procedures. *European Journal of Biochemistry* **191**, 389–396.
- ROUSSET, M., DERMOUN, Z., HATCHIKIAN, C. E. & BÉLAICH, J. P. (1990). Cloning and sequencing of the locus encoding the large and small subunit genes of the periplasmic [NiFe] hydrogenase from *Desulfovibrio fructosovorans*. *Gene* **94**, 95–101.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning, a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SAWERS, R. C. & BOXER, D. H. (1986). Purification and properties of membrane-bound hydrogenase isoenzyme 1 from anaerobically grown *Escherichia coli* K-12. *European Journal of Biochemistry* **156**, 265–275.
- SAWERS, R. G., BALLANTINE, S. P. & BOXER, D. H. (1985). Differential expression of hydrogenase isoenzymes in *Escherichia coli* K-12: evidence for a third isoenzyme. *Journal of Bacteriology* **164**, 1324–1331.
- SAYAVEDRA-SOTO, L. A., POWELL, G. K., EVANS, H. J. & MORRIS, R. O. (1988). Nucleotide sequence of the genetic loci encoding subunits of *Bradyrhizobium japonicum* uptake hydrogenase. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 8395–8399.
- SCHATZ, P. J. & BECKWITH, J. (1990). Genetic analysis of protein export in *Escherichia coli*. *Annual Review of Genetics* **24**, 215–248.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- UFFEN, R. L., COLBEAU, A., RICHAUD, P. & VIGNAIS, P. (1990). Cloning and sequencing the genes encoding the uptake-hydrogenase subunits of *Rhodocyclus gelatinosus*. *Molecular and General Genetics* **221**, 49–58.
- VAN DONGEN, W., HAGEN, W., VAN DEN BERG, W. & VEEGER, C. (1988). Evidence for an unusual mechanism of membrane translocation of the periplasmic hydrogenase of *Desulfovibrio vulgaris* (Hildenborough), as derived from expression in *Escherichia coli*. *FEMS Letters* **50**, 5–9.
- VAN DER WESTEN, H. M., MAYHEW, S. G. & VEEGER, C. (1978). Separation of hydrogenase from intact cells of *Desulfovibrio vulgaris*. *FEBS Letters* **86**, 122–126.
- VLASUK, G. P., INOUE, S., ITO, H., ITAKURA, K. & INOUE, M. (1983). Effects of the complete removal of basic amino acid residues from the signal peptide on secretion of lipoprotein in *Escherichia coli*. *Journal of Biological Chemistry* **258**, 7141–7148.
- VON HEIJNE, G. (1985). Signal sequences, the limits of variation. *Journal of Molecular Biology* **184**, 99–105.

- VOORDOUW, G. (1990). Hydrogenase genes in *Desulfovibrio*. In *Microbiology and Biochemistry of Strict Anaerobes Involved in Interspecies Hydrogen Transfer*, pp. 37–51. Edited by J. P. Bélaich, M. Bruschi & J. L. Garcia. New York: Plenum.
- VOORDOUW, G. & BRENNER, S. (1985). Nucleotide sequence of the gene encoding the hydrogenase from *Desulfovibrio vulgaris* (Hildenborough). *European Journal of Biochemistry* **148**, 515–520.
- VOORDOUW, G., HAGEN, W. R., KRÜSE-WOLTERS, M., VAN BERKEL-ARTS, A. & VEEGER, C. (1987). Purification and characterization of *Desulfovibrio vulgaris* (Hildenborough) hydrogenase expressed in *Escherichia coli*. *European Journal of Biochemistry* **162**, 31–36.
- VOORDOUW, G., MENON, N. K., LEGALL, J., CHOI, E. S., PECK, H. D., JR & PRZYBYLA, A. E. (1989*a*). Analysis and comparison of nucleotide sequences encoding the genes for [NiFe] and [NiFeSe] hydrogenases from *Desulfovibrio gigas* and *Desulfovibrio baculatus*. *Journal of Bacteriology* **171**, 2894–2899.
- VOORDOUW, G., STRANG, J. D. & WILSON, F. R. (1989*b*). Organization of the genes encoding [Fe] hydrogenase in *Desulfovibrio vulgaris* subsp. *oxamicus* Monticello. *Journal of Bacteriology* **171**, 3881–3889.
- VOORDOUW, G., NIVIÈRE, V., FERRIS, F. G., FEDORAK, P. M. & WESTLAKE, D. W. S. (1990). Distribution of hydrogenase genes in *Desulfovibrio* spp. and their use in identification of species from oil field environment. *Applied and Environmental Microbiology* **56**, 3748–3754.
- WATSON, M. E. E. (1984). Compilation of published signal sequences. *Nucleic Acids Research* **12**, 5145–5164.
- WONG, S.-L. (1989). Development of an inducible and enhancible expression and secretion system in *Bacillus subtilis*. *Gene* **83**, 215–223.