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

VINCENT NIVIÈRE,† SUI-LAM WONG and GERRIT VOORDOUW*

Division of Biochemistry, Department of Biological Sciences, The University of Calgary, Alberta, Canada T2N 1N4

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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...
**Sulphate-reducing bacteria**

<table>
<thead>
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**Nitrogen-fixing bacteria**

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<td>MSLRTETFYDMRRQGGIT RR S F L K YCSLTAAALGLPAFAPRIAHAMET</td>
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**Photosynthetic bacteria**

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<td>METFYEMRRQGIS RR S F L K YCSLTATGLPSFVPQIAHAMEMET</td>
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**Enterobacteria**

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<td>Ec</td>
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Consensus box: RR F K

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Fig. 1. Signal peptide sequences determined for small subunits of [Fe], [NiFe] and [NiFeSe] hydrogenases from different microorganisms. The sequences have been aligned relative to the consensus sequence RRXFXK. Positively (+; R, K) and negatively (−; E, D) charged amino acid residues are indicated. The following are the full names of the microorganisms and references to the sequences:

- **DvH [Fe]** is *Desulfovibrio vulgaris* Hildenborough (Voordouw & Brenner, 1985);
- **DvMo [Fe]** is *D. vulgaris* examicus Monticello (Voordouw et al., 1989);
- **Db [NiFeSe]** is *D. baculatus* (Voordouw et al., 1989a);
- **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., 1989a);
- **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., 1990a);
- **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., 1990b).

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: RRXFXK. 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 αβ 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 [2-35S]SAlATP (400 Ci mmol−1, 10 mCi ml−1; 1 Ci = 37 GBq) for dyeoxy-seqencing was from Amersham. Trans35S-label, containing 70% L-[35S]methionine and 15% L-[35S]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. PADC 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, 2 µM-sodium selenite and 30 mM-formate, 0.4% (w/v) glucose, 2 µM-sodium selenite and 30 mM-formate, 0.4% (w/v) glucose, pH 7.4, as described by Lee et al. (1990). Anaerobic membranes from Schleicher and Schuell. Reagent-grade chemicals were obtained from either Fisher, Sigma or Pharmacia.

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 (5 µg ml−1) or ampicillin (100 µg ml−1). Plasmid pKUL6 (Fig. 2) was obtained by digestion of pVN1 with HindIII and ScaI and ligation with a 546 nt HindIII-ScaI fragment from pBR322. A 1-38 kb DraI-NdeI fragment containing the

and dideoxynucleotide sequencing of M13 subclones (Sanger et al., 1977). Plasmid pVN0, expressing β-lactamase with the native signal peptide (Fig. 3b, v) from tandemly arranged lac and bla promoters, was obtained by digestion of pVN1 with HindIII and ScaI and ligation with a 546 nt HindIII-ScaI fragment from pBR322. Plasmid DV1, containing two ClaI sites, was partially digested with ClaI (note: only the ClaI site targeted for modification is shown in Fig. 2), end-repaired with Klenow polymerase and dNTPs and BamHI extension-ligation mixtures into pUC-4K (Wong, 1989), expressing a β-lactamase from the lac promoter, was verified by restriction digestion

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

<table>
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<tr>
<th>Strain/vector</th>
<th>Genotype, comments and reference</th>
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<tr>
<td>E. coli TG2</td>
<td>Δlac-pro supE thi hsdM rcsA F' (τD61 proAB* lacZAM15). (from T. J. Gibson, MRC Centre, Cambridge, UK)</td>
</tr>
<tr>
<td>E. coli TG108</td>
<td>As E. coli TG2 but lacking F’ (this study)</td>
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<tr>
<td>E. coli RZ1032</td>
<td>Hfr KL16 PO/45 (lysA61-62) dUT-1 ung-1 thi-1, relA1 Zbd-279: Trn10 supE44 (Kunkel et al., 1987)</td>
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<tr>
<td>DV1</td>
<td>Contains the structural genes for the [NiFe] hydrogenase from D. vulgaris Hildenborough in bluescript; ApR (from A. E. Przybyla, University of Georgia, USA)</td>
</tr>
<tr>
<td>PGV21</td>
<td>Derived from DV1; ApR (this study)</td>
</tr>
<tr>
<td>PGV32</td>
<td>Derived from pGV21; ApR (this study)</td>
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<tr>
<td>pKUL6</td>
<td>Contains the levansucrase signal peptide gene fused in-frame to the gene of mature β-lactamase; KmR (this study)</td>
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<tr>
<td>pKUL7</td>
<td>Derived from pKUL6; contains the gene for mature β-lactamase without signal peptide; KmR (this study)</td>
</tr>
<tr>
<td>pVN0</td>
<td>Derived from pKUL7; contains the wild-type bla gene; KmR (this study)</td>
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<td>pIP101</td>
<td>Contains the lacZ gene; ApR (from B. Muller-Hill, University of Cologne, Germany)</td>
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<tr>
<td>pWB</td>
<td>Obtained from Kunkel et al., 1987</td>
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<td>pBR322, pUC19, M13mp18, pUC-4K</td>
<td>Purchased from Pharmacia (Sambrook et al., 1989)</td>
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supplemented with 50 µg kanamycin ml\(^{-1}\) and shaken at 250 r.p.m. in a New Brunswick G25 shaker for the determination of \(\beta\)-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 µg L-proline, 1 µg of thiamin and 50 µg 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 µl Tran\(^{35}\)S-label (10 mCi µl\(^{-1}\)). Chase was initiated by addition of 50 µl 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 µl 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 µl portion of a solubilized sample was added to 1 ml Triton buffer (Ito et al., 1981) together with 8 µl purified polyclonal anti-\(\beta\)-lactamase antibodies (0.6 mg ml\(^{-1}\)). Purification was by immuno-affinity chromatography using \(\beta\)-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 µl SDS loading buffer [10% (w/v) SDS, 250 mM-Tris/HCl, pH 6.8, 50% (w/v) glycerol, 0.5% (w/v) bromo-
tetrazolium and dary antibody and the blot was immunostained with nitroblue directly to 1 ml samples of exponential-phase aerobic or anaerobic TY 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 gene encoding lac repressor is deleted from this strain (Table 1).
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(pVNO), 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(pVNO). 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).
Hydrogenase signal peptide

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. 3b, 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. 3b, 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. 3b, iv and v)
Fig. 5. Effect of mutation of signal peptide residue R(-28) on the processing of the $\beta$-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 $\beta$-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 $\beta$-lactamase are explained in the legend to Fig. 4. Pre $\beta$-lact and Mat $\beta$-lact represent the precursor and the mature forms of $\beta$-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 $\beta$-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 $\beta$-lactamase activities measured for the R(-28) mutants are summarized in Table 2. The R(-28)E mutation strongly affected the export of $\beta$-lactamase to the periplasm. Compared to the wild-type signal peptide, we observed an 84-93% decrease of periplasmic $\beta$-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-$\beta$-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-$\beta$-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 (RRXXXR) is similar to that on the COOH-terminal side of R(-28), which is RRXXXK (Fig. 3b). Compared to the wild-type signal peptide, mutations R(-33)E, R(-33)M and R(-33)V decreased the export of $\beta$-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

<table>
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<td>0.55</td>
<td>100</td>
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<td>0.12</td>
<td>0.18</td>
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<td>R(-28)V</td>
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<td>Anaerobic</td>
<td>0.15</td>
<td>0.57</td>
<td>104</td>
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* $\beta$-Lactamase activity is expressed in mmol PADAC min$^{-1}$ (10$^8$ cells)$^{-1}$.
† This includes both cytoplasmic and membrane-bound activities.
‡ Ratio [(periplasmic activity)/(periplasmic activity of wild-type)] x 100.
§ Data for *E. coli* TG108(pKUL7).
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 c1. 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. 3b, 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 mutated, 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

sequencing of a [NiFe] hydrogenase operon from Desulfovibrio
gigas. FEMS Microbiology Letters 75, 203–208.

FAUQUE, G., PECK, H. D., JR., MOURA, J. J. G., HUYNI, B. H.,
BERLIER, Y., DERVARTANIAN, D. V., TEIXEIRA, M., PRZYBYLA,
classes of hydrogenases from sulfate reducing bacteria of the genus
Desulfovibrio. FEMS Microbiology Reviews 54, 299–344.


in E. coli: is there a common step in the secretion of periplasmic and


efficient site-specific mutagenesis without phenotypic selection.

685.

Cloning and sequencing of the genes encoding the large and the small
subunits of the H2 uptake hydrogenase (hup) of Rhodobacter
capsulatus. Molecular and General Genetics 214, 97–108.

a new gene, molR, essential for utilization of molbdate in

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, 10380–10383.

Lutz, S., Jacob, A., SCHLENZOG, V., BÖHM, R., SAWERS, G. & BÖCK,
coli. Molecular Microbiology 5, 123–135.

Menon, N. K., ROBBINS, J., WENDT, J. C., SHANMUGAN, K. T. &
Escherichia coli hyp operon, which encodes [NiFe] hydrogenase 1.
Journal of Bacteriology 173, 4851–4851.


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. &
c1 from Desulfovibrio vulgaris Hildenborough in Escherichia coli:
export and processing of the apoprotein. Journal of General
Microbiology 135, 2319–2328.


PUZINS, 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.

RAMIREZ, M., FURSTENAU, U., MAYER, F., PRZYBYLA, A. E., PECK,

ROUSSET, M., DERMOUN, Z., HATCHIKIAN, C. E. & BÉLAICH, J. P.
(1990). Cloning and sequencing of the locus encoding the large and

Cold Spring Harbor Laboratory.

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.

expression of hydrogenase isoenzymes in Escherichia coli K-12:
evidence for a third isoenzyme. Journal of Bacteriology 164, 1324–
1331.

(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.


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.

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. & Veejer, C.
(1988). Evidence for a novel mechanism of membrane transloca-
tion of the periplasmic hydrogenase of Desulfovibrio vulgaris
(Hildenborough), as derived from expression in Escherichia coli.
FEMS Letters 50, 5–9.

Separation of hydrogenase from intact cells of Desulfovibrio vulgaris.
FEMS Letters 86, 122–126.

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.


