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A regulatory domain controls the transport activity of a twin-arginine signal peptide

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ABSTRACT

The twin-arginine translocation (Tat) pathway is used by bacteria for the transmembrane transport of folded proteins. Proteins are targeted to the Tat translocase by signal peptides that have common tripartite structures consisting of polar n-regions, hydrophobic h-regions, and polar c-regions. In this work, the signal peptide of [NiFe] hydrogenase-1 from *Escherichia coli* has been studied. The hydrogenase-1 signal peptide contains an extended n-region that has a conserved primary structure. Genetic and biochemical approaches reveal that the signal peptide n-region is essential for hydrogenase assembly and acts as a regulatory domain controlling transport activity of the signal peptide.

1. Introduction

Molecular hydrogen (H_2) is widely used as a respiratory electron donor by microbes. *Escherichia coli* synthesises two periplasmically-oriented but membrane-bound [NiFe] hydrogenases – Hyd-1 and Hyd-2 [1]. The Hyd-1 isoenzyme is of particular interest since it is a well-characterised example of an O_2-tolerant hydrogenase that can protect itself, and recover enzymatic activity, following oxygen attack [2]. The crystal structure of *E. coli* Hyd-1 has recently been solved and it has been shown to comprise an α-subunit containing the [NiFe] active site and a β-subunit containing three [Fe–S] clusters [3]. The final step in Hyd-1 biosynthesis is its translocation from the cytoplasm to the periplasm. This process is catalysed by the twin-arginine translocation (Tat) system [4]. The Hyd-1 β-subunit is synthesised as a precursor with an N-terminal twin-arginine signal peptide. The structural characteristics of a Tat signal peptide include a polar 'n-region', a central hydrophobic 'h-region', and polar 'c-region' containing a signal peptidase cleavage site [4]. The conserved S/TRRxFLK twin-arginine amino acid motif is always between the n- and h-region [4]. [NiFe] hydrogenase signal peptides are notable in that have greatly extended n-regions and exhibit remarkable sequence conservation [5].

In this work, the role of the *E. coli* Hyd-1 signal peptide n-region has been investigated. Genetic removal of the signal peptide n-region was found to have a negative impact on the biosynthesis of Hyd-1. When studied in isolation, the full length Hyd-1 Tat signal peptide was found to export a reporter protein only inefficiently in comparison to a truncated signal peptide. Finally, site-directed mutagenesis identified residues within the n-region that are important in signal peptide activity. Taken altogether, it is concluded that the signal peptide n-region operates as an important regulatory domain that can modulate signal peptide activity during biosynthesis of Hyd-1.

2. Materials and methods

2.1. Bacterial strains

Strain IC009 Y8W was constructed as follows: DNA surrounding the *hydA* n-region was amplified by PCR and cloned into pBluescript KS^+. The Y8 codon was substituted with one for tryptophan by Quikchange™ before the fragment was moved onto pMAK705 [6] and onto the chromosome of *E. coli* K-12 strain IC009 (as MC4100, Δ*hybC*, Δ*hycE*) [7]. Strain IC009ΔNR was generated as follows: approximately 1000 bp of DNA surrounding the 5′ end of *hyaA* (lacking the sequence encoding the signal peptide n-region N2–V16) was

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synthesised by Biomatik Corp and cloned into pMAK705. The Δn-region allele was then transferred onto the chromosome of IC009.

2.2. Plasmids, site-directed mutagenesis and RT-PCR

Plasmid pUniCAT-HyaA and pUniCAT-HyaAΔN were constructed by PCR-amplifying DNA encoding the full-length HyaA signal peptide, and a truncated version lacking N2–V16, and cloning those products separately into pUNI-REP [8]. A library of 15 tryptophan codon substitution mutants were prepared by subjecting pUniCAT-HyaA to QuikChange site-directed mutagenesis. The pQE80-HyaA overproduction plasmid was generated by first cloning *hyaA*, as well as part of *hydB*, into pUNI-PROM [9] and then moving an *Eco*RI–*Hind*III fragment (including the *tat* promoter sequence) into pQE80L (Qiagen).

For RT-PCR, total RNA was extracted from IC009 to IC009ΔNR anaerobic cultures in the stationary phase of growth using the RNeasy minikit (Qiagen). The cDNA was synthesised from 500 ng RNA using Superscript III reverse transcriptase (Invitrogen), random hexamer primers and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen) and then incubated at 50 °C for 50 min. RNase H was added to digest any residual RNA template and the mixture incubated at 37 °C for 20 min. Oligonucleotides designed to amplify regions of *hyaA*, *hyaB* and *hyaE* were used for PCR analysis of the generated cDNA.

2.3. Protein analysis and immunoelectrophoresis

Chromosomally-encoded Hyd-1^{His} was purified from *E. coli* FTH004 (as MC4100, *hyaA*^{His}) as previously described [1]. Antisera were raised in rabbits by Eurogentec. For subcellular fractionation the method of Osborn & Munson was followed [10]. SDS–PAGE was by Lämmli [11] and Western immunoblotting by Dunn [12]. For rocket immunoelectrophoresis, membranes fractions were first solubilised in 50 mM Tris–HCl pH 7.6 containing 4% Triton X-100 (v/v). Samples were added to small wells of a 1% (w/v) agarose gel containing electrophoresis buffer (20 mM sodium barbitone–HCl, pH 8.6, 0.1% (v/v) Triton X-100) and, Hyd-1 specific antiserum diluted 1 in 10 (5 μl/3 ml gel). Samples were electrophoresed at 2 mA per plate for 16 h. Plates were then removed, immersed in 50 mM Tris–HCl buffer containing benzyl viologen (BV) and Tetrazolium Red, and incubated under an atmosphere of 100% H_2 for 16 h.

2.4. Pulse-chase transport assays

E. coli strain K38[pGP1-2] [13], which is *tat*$^+$ and considered a good host strain for radiolabelling experiments [14], was transformed separately with pUniCAT-HyaA, pUniCAT-HyaAΔN and their derivatives and whole cells pulse-labelled with 50 μCi of ^{35}S methionine for 2 min before being chased with unlabelled methionine (0.75 mg/ml) essentially as described [14]. Samples (0.5 ml) were withdrawn at 0, 0.5, 1, 2, 5, 15, 30 and 60 min intervals, separated by SDS–PAGE and analysed following autoradiography. Each pulse-chase was performed in triplicate and band intensity quantified using the QuantityOne software package (Bio-Rad).

2.5. Antibiotic sensitivity assays

The *E. coli* K-12 parent strain MG1655 (*tat*$^+$) [15] was transformed with the appropriate plasmid and grown in LB containing ampicillin, and either 100 μg/ml or no added chloramphenicol, before growth was followed in a 96 well plate format in a Biotek 2 shaking incubator platereader, essentially as described [8,16].

3. Results

3.1. The signal peptide n-region is critical for Hyd-1 assembly and activity

Tat signal peptides of oxygen-tolerant [NiFe] hydrogenases share sequence conservation (Fig. 1). In addition to the twin-arginine motif, there are conserved aromatic, cysteine and polar residues within the n- and h-regions (Fig. 1). To investigate the role of the *E. coli* Hyd-1 signal peptide n-region, an in-frame deletion allele (removing *hyaA* codons 2–16) was generated and incorporated onto the chromosome of strain IC009 (as MC4100, Δ*hybC*, Δ*hycE*), which produces Hyd-1 as the only active hydrogenase, to give strain IC009ΔNR (as MC4100, Δ*hyaA2–16*, Δ*hybC*, Δ*hycE*). The new strain IC009ΔNR was grown anaerobically and membranes prepared before the presence of active Hyd-1 was assessed using rocket immunolelectrophoresis (Fig. 2) [17]. In this technique, non-denatured samples are electrophoresed through an agarose gel containing anti-Hyd-1 serum. The resultant arcs of precipitin can then be stained for hydrogenase activity and their relative sizes give an indication of the amount of Hyd-1 present in each sample. In this experiment, active Hyd-1 was detected in the membranes of IC009 (Δ*hybC*, Δ*hycE*), but no detectable Hyd-1 activity was observed in the membranes of IC009ΔNR (Δ*hyaA2–16*, Δ*hybC*, Δ*hycE*) (Fig. 2A).

Western immunoblotting identified the Hyd-1 α-subunit (HyaB) in the membrane fraction of the parental strain IC009, as well as in the cytoplasm (Fig. 2C). In contrast, the catalytic α-subunit HyaB was only weakly detectable in the cytoplasm of IC009ΔNR (Δ*hyaA2–16*, Δ*hybC*, Δ*hycE*) and was completely absent from the membrane fraction (Fig. 2C), thus corroborating the rocket immunoelectrophoresis experiment (Fig. 2A). The Hyd-1 β-subunit (HyaA) was clearly present in the membrane fraction of IC009 (Fig. 2D). However, the β-subunit HyaA was not detectable at all in the n-region deletion strain (Fig. 2C). To address if the reduced levels of Hyd-1 enzyme observed in the IC009ΔNR (Δ*hyaA2–16*, Δ*hybC*, Δ*hycE*) strain were as a result of a problem with *hya* transcription in this strain, RT-PCR was utilised (Fig. 2E). RNA was extracted from both IC009 and IC009ΔNR and RT-PCR performed using primers designed to identify *hyaA*, *hyaB* and *hyaE* transcripts (Fig. 2E). Although this RT-PCR technique is not quantitative and cannot report on relative levels of *hya* transcription between strains, it can be concluded from these experiments that entire *hya* operon is transcribed in the IC009ΔNR (Δ*hyaA2–16*, Δ*hybC*, Δ*hycE*) strain.

To complement the n-region deletion phenotype in trans DNA encoding the entire *hyaA* gene, together with a constitutive *tat* promoter, was cloned into the IPTG-inducible vector pQE-80L. Detergent-dispersed membrane proteins were obtained from

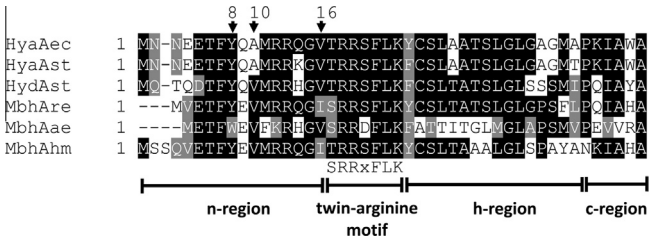

Fig. 1. Sequence conservation in O_2-tolerant hydrogenase signal peptides. Sequences are the Tat signal peptides of *E. coli* Hyd-1 (HyaAec), *Salmonella* Hyd-1 (HyaAst) and Hyd-5 (HydA st), *R. eutropha* MBH (MbhAre), *A. aeolicus* (MdhAae) and *Hydrogenovibrio marinus* (MbhAhm). The arrows indicate the positions of *E. coli* HyaA residues Y8, A10 and V16 that are found to be important for signal peptide activity in this study.