Cloning of the *Rhodobacter sphaeroides* *hisI* gene: unfunctionality of the encoded protein and lack of linkage to other *his* genes

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The *Rhodobacter sphaeroides* 2.4.1 *hisI* gene, which encodes a phosphoribosyl-AMP-cyclohydrolase that catalyses the third step in the histidine biosynthetic pathway, has been isolated from a genomic library of this phototrophic bacterium by complementation of an *Escherichia coli* *hisI* mutant. Analysis of the nucleotide sequence of the *R. sphaeroides* *hisI* gene reveals that it encodes a deduced product of 119 aa with a predicted molecular mass of 13.4 kDa. In contrast to the situation in *E. coli*, the *R. sphaeroides* *hisI* gene encodes a unifunctional protein and it is not linked to the *hisE* gene. The absence of a single histidine operon like that of *E. coli* was confirmed by PFGE experiments and complementation analysis of a *R. sphaeroides* *hisI* mutant that was constructed by marker exchange. The location of *hisI* in the *R. sphaeroides* genome has been determined to be at map co-ordinate 2275 ± 20 of chromosome I.

Keywords: *hisI* gene, *Rhodobacter sphaeroides*, histidine biosynthesis, chromosome mapping

INTRODUCTION

The biosynthetic pathway for histidine has been widely studied in different micro-organisms, both prokaryotes and eukaryotes. Although the intermediate products and the enzymes involved seem to be quite similar for diverse organisms, there are important differences among them in terms of gene distribution and regulation mechanisms.

The biosynthesis of histidine is carried out by an unbranched pathway that is more closely linked to the metabolism of pentoses and purines than to any of the other amino acids. This pathway has been analysed in *Escherichia coli* and *Salmonella typhimurium* both biochemically (Martin *et al.*, 1971) and genetically (Carlomagno *et al.*, 1988). It is known that in these micro-organisms the eleven steps of the pathway are catalysed by enzymes encoded by eight structural genes, which are clustered in a single 7.5 kb operon, organized in the sequence *hisGDCBHAF(IE)*. A primary promoter and two internal promoters (to avoid the polar effect), as well as a rho-independent terminator, have been identified (Winkler, 1987).

Studies in other organisms show that the arrangement of

his genes is very variable. In *Lactococcus lactis* (Delorme *et al.*, 1992), the relative order of the genes is the same as in enteric bacteria, but the operon contains additional ORFs of unknown function. In *Streptomyces coelicolor* (Limauro *et al.*, 1990) several genes are located in a main cluster, while other genes have been found in unlinked loci of the chromosome. A main cluster that does not contain all the genes is also found in *Bacillus subtilis* (Henner *et al.*, 1986) and *Azospirillum brasilense* (Fani *et al.*, 1993). In *Saccharomyces cerevisiae* (Donhaue *et al.*, 1982) and *Neurospora crassa* (Legerton & Yanofsky, 1986) the histidine genes are scattered over different chromosomes.

The histidine biosynthetic pathway is extensively used for studies of gene structure, expression and regulation. *E. coli* and *S. typhimurium* possess an elaborate network to control the rate of histidine biosynthesis, which includes feedback inhibition, metabolic regulation, attenuation and control by supercoiling (Winkler, 1987; O'Byrne *et al.*, 1992). The wide information acquired on *his* genes allows us to use them as a model system for the study of gene regulation.

To the best of our knowledge there are only two reported sequences of genes related to the biosynthesis of amino acids (*trpC* and *glnAB*) from photosynthetic organisms (Becker-Rudzik *et al.*, 1992; Zinchenko *et al.*, 1994), and no histidine gene has yet been characterized. The isolation

The EMBL accession number for the nucleotide sequence reported in this paper is X82010.

of the *R. sphaeroides* histidine genes would be useful, not only to analyse the metabolic pathway of this amino acid further, but also to provide a model for the study of regulation of biosynthetic genes in a phototrophic bacterium.

As a first step to studying the regulation mechanisms of histidine biosynthesis in *R. sphaeroides*, the *hisI* gene was isolated by complementation of a characterized mutant of *E. coli*. This technique takes advantage of the fact that the histidine biosynthesis genes of different species can complement auxotrophic strains of *E. coli*, even if they are not phylogenetically related (Bazzicalupo *et al.*, 1987).

The *E. coli hisIE* gene, which is 610 bp in size, is located in the distal part of the histidine operon and encodes a phosphoribosyl-ATP-pyrophosphohydrolase:phosphoribosyl-AMP-cyclohydrolase, which catalyses the second and third steps in the histidine biosynthetic pathway. These include hydrolysis of *N*'-(5'-phosphoribosyl)-ATP to *N*'-(5'-phosphoribosyl)-AMP and pyrophosphate (*hisE* enzyme activity), followed by a ring-opening reaction on the purine (*hisI* enzyme activity).

In this paper, we report the cloning and sequencing of the *hisI* gene, as well as its mapping on the chromosome of *R. sphaeroides* and the construction of a *hisI* mutant. Surprisingly, unlike most micro-organisms studied so far, we have found that the *R. sphaeroides hisI* gene encodes a unifunctional protein and that it is not linked to the *hisE* gene.

METHODS

Bacterial strains and plasmids. These are listed in Table 1.

Media and growth conditions. *E. coli* and *S. typhimurium* strains were grown in either complex LB (Miller, 1992) or NB medium (Nutrient Broth no. 2; Oxoid), or minimal AB medium supplemented with 0.2% (w/v) glucose (Clarke & Maaløe, 1967) at 37 °C for 16–20 h. Plasmid extracts were made from cultures growing in TB medium (Sambrook *et al.*, 1989).

R. sphaeroides strains were grown in complex Z broth (Pemberton & Bowen, 1981) or minimal Pfennig medium (Pfennig & Trupper, 1983) lacking yeast extract and supplemented with 0.2% (w/v) sodium succinate. In all cases, growth of *R. sphaeroides* was performed aerobically in the dark at 30 °C for 36–48 h.

Media were supplemented when necessary with 25 µg histidine ml⁻¹, 40 µg X-Gal ml⁻¹ or antibiotics at the following concentrations: 17 µg tetracycline ml⁻¹ for *E. coli* and 1.7 µg tetracycline ml⁻¹ for *R. sphaeroides*; 50 µg ampicillin ml⁻¹; 34 µg chloramphenicol ml⁻¹; 75 µg rifampicin ml⁻¹; 75 µg streptomycin ml⁻¹; 100 µg spectinomycin ml⁻¹.

Construction of a *R. sphaeroides* library. *R. sphaeroides* chromosomal DNA was extracted, partially digested with *Sau3A*I and purified by ultracentrifugation on a sucrose gradient (Ausubel *et al.*, 1989). Fragments of 5–8 kb were ligated to the broad-host-range cloning vector pRK404 (Ditta *et al.*, 1985), previously digested with *Bam*HI. The ligation mix was introduced into *E. coli* DH5α by electroporation (Dower *et al.*, 1988), and cells were plated on LB agar supplemented with tetracycline and X-Gal. The library was amplified and maintained at –70 °C in 15% (w/v) glycerol.

Molecular biology techniques. Plasmid DNA isolation, agarose gel electrophoresis, restriction endonuclease digestions, enzyme treatment and transformation were carried out as described elsewhere (Sambrook *et al.*, 1989). Random primer labelling and Southern blot hybridization were performed following the 'DIG System User's Guide for Filter Hybridization' from Boehringer Mannheim.

Prior to DNA sequencing, a set of exonuclease III-mediated nested deletions of the DNA fragment of interest was obtained, using the Erase-a-Base system (Promega). The DNA sequence was determined for both strands by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) on an ALF Sequencer (Pharmacia Biotech) or on a GATC 1500-System DNA sequencer (MWG-Biotech), using M13 primers of pBluescript SK(+) in both cases.

Nucleic acid and protein sequences were analysed using the University of Wisconsin Genetics Computer Group software package, version 8.0.

Pulsed-field gel electrophoresis. Intact genomic DNAs from *R. sphaeroides* were prepared in InCert agarose plugs as described by Smith & Cantor (1987). PFGE was performed in a Pharmacia-LKB apparatus. Gels were made of 1% (w/v) agarose (SeaKem LE; FMC) and run at 15 °C in modified TBE buffer (100 mM Tris, 100 mM boric acid, 0.2 mM EDTA; final pH 8.0–8.4). Electrophoresis conditions were standardized in order to obtain two different resolution windows: 10 V cm⁻¹ for 36 h, with a 10–120 s pulse ramp, resolved fragments from 200 to 1600 kb; 6.6 V cm⁻¹ for 24 h, with a 5–35 s pulse ramp, resolved fragments from 30 to 450 kb. Chromosomes of *S. cerevisiae* YPH80 (Promega) and phage λ DNA concatamers (New England Biolabs) were used as size standards.

RESULTS AND DISCUSSION

Cloning of the *R. sphaeroides hisI* gene

To isolate the *R. sphaeroides hisI* gene, a genomic library of this bacterium, constructed as described in Methods, was transferred from *E. coli* DH5α to *E. coli* UA4845 (*hisI*) by triparental mating, using the plasmid pRK2013 as helper. Transconjugants that grew in the absence of exogenous histidine on plates containing tetracycline and rifampicin were selected. Although *E. coli* and *R. sphaeroides* are not closely related, the *hisI* genes are conserved enough to encode functionally equivalent proteins.

Plasmid pUA509, containing a 4.9 kb insert, was isolated from one of these transconjugants and further characterized. First, it was introduced by transformation into various *E. coli his* mutants, namely *hisA*, *hisB*, *hisC*, *hisD*, *hisF* and *hisG*, in order to test its ability to complement histidine mutations other than *hisI*. No complementation was observed in any case. This plasmid was also introduced by electroporation into two different *hisI* strains of *S. typhimurium* (SB4281 and I125) in order to test complementation of pUA509 in different genetic backgrounds. In both cases, pUA509 allowed the recovery of the prototrophic phenotype.

An accurate restriction analysis of pUA509 showed that three fragments (0.9, 1.2 and 1.3 kb) were generated after digestion with *Sa*I. Each of these fragments was subcloned in the *Sa*I site of pBluescript SK, and the constructs were used to transform UA4845. Replica-

Table 1. Bacterial strains and plasmids used in this study

Strain	Description	Source or reference
<i>Escherichia coli</i>		
DH5 α	<i>deoR endA1 gyrA96 recA1 relA1 hsdR17</i> ($\tau_K^- m_K^-$) <i>supE44 thi-1</i> Δ (<i>lacZYA-argF</i>) ϕ 80 <i>lacZ</i> Δ M15 F $^-$ λ^-	Hanahan (1983)
UTH903	<i>hisI903 ara-14 galKZ</i> λ^- <i>rpsL145 malT1 xyl-5 mtl-1</i>	Goldschmidt <i>et al.</i> (1970)
UA4845	UTH903 Rf r	This study
<i>Salmonella typhimurium</i>		
SB4281	<i>hisIa99</i>	Hartman <i>et al.</i> (1971)
I125	<i>hisI125</i>	Hartman <i>et al.</i> (1971)
E311	<i>hisE311</i>	Hartman <i>et al.</i> (1971)
UA1701	<i>hisE311</i> Rf r	This study
<i>Rhodobacter sphaeroides</i>		
2.4.1	Wild-type	Kaplan, S. (University of Texas)
UA8002	Derivative of 2.4.1, Rf r	This laboratory
UA8123	Derivative of 2.4.1, Rf r Sp r Sm r HisI $^-$	This study
Plasmids		
pRK404	Mob $^+$ Tc r	Ditta <i>et al.</i> (1985)
pRK2013	Mob $^+$ Tra $^+$ Km r	Ditta <i>et al.</i> (1985)
pBluescript SK+	Ap r	Stratagene
pSUP202	Ap r Tc r Cm r	Simon <i>et al.</i> (1983)
pHP45 Ω SpSm	Ap r Sp r Sm r	Fellay <i>et al.</i> (1987)
pUA509	pRK404 carrying a 4.9 kb <i>R. sphaeroides</i> DNA fragment containing the <i>hisI</i> gene	This study
pUA510	pBluescript SK carrying a <i>SalI</i> – <i>SalI</i> 1.3 kb DNA fragment from pUA509	This study
pUA534	pSUP202 carrying a <i>BclI</i> – <i>BglI</i> 0.5 kb fragment from pUA510, disrupted by a Ω Sp/Sm interposon	This study
pUA556	pRK404 carrying a 8.8 kb <i>R. sphaeroides</i> DNA fragment containing the <i>hisE</i> gene	This study

Fig. 1. (a) Restriction map of the pUA510 insert and relative position of ORF1 and *hisI*. Solid boxes indicate the pBluescript SK polylinker. Only relevant sites are shown. (b) Ability of different fragments to complement *E. coli* and *R. sphaeroides* *hisI* mutants. Subclones were obtained either by the restriction enzymes indicated, or by exonuclease III-mediated deletions. Cloning vectors are shown in parentheses and the position of the *lac* promoter is indicated by an arrow. Only fragments cloned in pRK404 were tested in *R. sphaeroides* because pBSK is not stable in this organism (NT, not tested).

plating of several recombinant clones of each type on minimal medium, with and without histidine, showed that only the plasmid harbouring the 1.3 kb fragment (pUA510) complemented the mutation (Fig. 1a). Furthermore, cloning of this fragment in the opposite

orientation in relation to the *lac* promoter of the vector also enabled the transformed UA4845 to grow in the absence of histidine. This result indicates that there is a *R. sphaeroides* promoter in the 1.3 kb fragment, which can be expressed in *E. coli*.

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1  CGACCGTCC TGAGTCTCTG CTGTCGCCAC GGTATGTCTT CTTAAATCC CGTTCGGTTC 60
61  TTAAGAAAGT CTTTACACTG GTTACCGGTT GTGGGAACAA TGACGGAACA TAAAGACGGA 120
121  GATCGCTCTT GTGACCGAG ATGAAGGAAG TCTCTCCCG CTGCTCCGAG ACACTGATCG 180
181  AGGATGCGCT TGGCTGGCT GCGCTCTTTT CTCTTCTGAT CGCGCGGCTC TACCTGCGGC 240
241  ACCTCGTCTG CCGTCCGAGC CGCCTGTGCT CTGTCGCCCA AGGATCCCCA GGACCGGAGC 300
301  CCACTGCGCG CGCCCTTTCG CCGCGGCGGC GCGCTTTTCA TGCGGGCTGG CCGGTAGAGG 360
361  CAGCGTGGGG CGGTTCTGCA GCGTTTATCG GCGAGGGGCA AGGCTTCGGC CGCGCTTCC 420
421  AGCGCGAGCA GGATGGAGC GTGCGGGTTG CGGTAGTGGC GCGCGGCGAG GAGCACCTCG 480
481  TAGCCCTCGA AGGGGCGGCG CGGCACCGGC CCGGCTTCGG TCAGCATGGC CTTCAATGGC 540
541  TCGCGTGGCG CCTCGACCTC GCGCGCGGAG CGCGCGAGCA GCGCACCTCC CATGATCGAG 600
601  GCGGCGGCTT GACCCAGGCG GCAGGCGCTT ACATCTGGCG CGAATTCGGC CACCGCGCGC 660
661  TCCGCGAGGT TCAGATCCAC GGTACGCTTC GAGCGCGAGA CCGCGGAGCG CTTCCGCGCG 720
721  CTGCGCTGTG GGTGCGCAAG CCGCGCGGATG TCGCGGATG TCGCGGATG 780
781  CGCCCGAGAT AGAGGCTGAT CAGTGTGCTG TCGCTCATGG CTTTCTCTCT TCGCGTCGAC 840
841  CTCTAGATA GGCCTCCGCG CGTCCGATGG AAAGGAACCC GAGTCTCTCT TCGACCCGCG 900
901  ATCGCTCAAC TTGTATGCA ATGGCTCAT CCGCGCGGTC GCACAGGACC ATGCGACCGG 960
961  CGAGGTGCTG ATGATGGCTT GGATGAACGC CGAGCGCGTC GCGCGCACCG TCGAGACCGG 1020
1021  GTGCGTCACC TACTGCTGCG GCTCGCGGCA GGCCTTCTGG GTGAAGGGCG AGACGTCGGG 1080
1081  CCATGTCCAG CGCGTATGCT AGCTGCGGAT CGACTCGGAC CGCGATGCGC TCGTCTGCTT 1140
1141  CATCGAGCAG GAGGCGCGCG CGTCCCATAC CAACGCGCGC TCGCTCTCTT ACACGCGGCT 1200
1201  CCGCGAGGCG GAGGAGCGGA TCATCTCGCA CCGGATGCTC TGACCGCGCT CGCGGCGGCG 1260
1261  TCCGCGCCAT CTGATTCGCG GTCTAGGTCC GCGCGGGGCG CAGGTGCGAA CGCTGAGCC 1320
1321  CGGTGCGAC 1328

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Fig. 2. Nucleotide sequence of the *Sa*II-*Sa*II fragment of pUA510. The deduced amino acid sequence is shown for ORF1 and *hisI*. Translational start codons are boxed, and arrows above them show the direction of transcription. Asterisks indicate translational stop codons. The putative RBSs preceding the predicted translational start codons are underlined. The -10 and -35 regions of the putative σ^{70} -like promoter located upstream of ORF1 are overlined.

In order to confirm that the cloned fragment belonged to the *R. sphaeroides* chromosome and that no reorganizations had taken place during the cloning process, a 0.7 kb *Eco*RI-*Hind*III fragment obtained from pUA510 was used as a probe to hybridize with total *R. sphaeroides* DNA digested with *Sa*II. As expected, a hybridization band of 1.3 kb was detected (data not shown).

Nucleotide sequence analysis of *hisI*

The nucleotide sequence of the 1.3 kb fragment contained in pUA510 was determined (Fig. 2). Two ORFs were found; the overall G+C content of the sequence is 67.3 mol %. This percentage, as well as the codon usage of the gene products, clearly biased towards G and C in the third position of the codon, is in agreement with the data from other known *Rhodobacter* genes.

ORF1 and its putative protein product show no significant homology with any other DNA or amino acid sequences

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1  #IVQNMDLKF RKIDDKELLI ALAIDRYN. VLMTAPMKR SLRNLTKGL MHYFSRNRK 60
1  #ISTILSSVN KSDRPDGLLI TVVVDH. DT ALGLVSSAA RVNRLTQT GYQSRKR.G
1  #TRVVLGEVR .TDRPDGLIT TVVVDY. ER CLGLVSSAA SLRAIDLGR GYVSRRE
1  ... NRDF H . KGLIP TVVVDYQNL VMLATNVE AFEMLETGE TWFSRSRK
1  MLTQQRREL DWERTDGLM VIVQVAVSGE VMLGTHNPE ALDRTIESGR VTFPSRTQR
1  MLTQQRREL DWERTDGLM AVQVAVSGE VMLGTHNPE ALDRTIESGR VTFPSRTQR
1  MLTQQRREL DWERTDGLM VIVQVAVSGE VMLGTHNPE ALDRTIESGR VTFPSRTQR
1  NAFDPASLTF D...ANGLIP AVAQDHATGE VLMHAMHAE AVARTVETGC VTYWRSRQA
1  -----L-----I-----L-L-Y-----G-----FSR-----
1  M. vannielii
1  N. crassa
1  S. cerevisiae
1  L. lactis
1  S. flexneri
1  S. typhimurium
1  E. coli
1  R. sphaeroides
1  Consensus
61  IWMKEESKN VQRVLEVPD CGDGLLFTV EQTQNA.CHE GYMSCFNKN DLTNGTSTVI 120
61  LWKGTSGD TQELVRISLD CNDILKRVV KQK.RFCHL DQSSCFQGLR .G.....
61  LWKGTSGN QRLQISTD CSDALKETV EQRVFCHL EYMSCFEGR HG.....
61  LWKGTSGN FQKTKWGLD CQDZLLVTV EQTQNA.CHT GAYSCFD... ELIPDSDSI
61  LWKGTSGN FLNVVSIAP CNDITLLVA NPIGPT.CHK GTSSCFGN... T...AHQWLF
61  LWKGTSGN FLNVVSIAP CNDITLLVA NPIGPT.CHK GTSSCFGN... A...SHOWLF
61  LWKGTSGN FLNVVSIAP CNDITLLVA NPIGPT.CHK GTSSCFGN... T...AHQWLF
61  FWKGTSGH VQRLIELRID CDRCLLLLI EQEPA.CHT NRSCFYTT... ALRGEERII
61  -W-KGE-SG-----D-CD-D-L-----G-CH--SCF-----
121  GDRLD*..... 180
121  LPKLEQTLIS RKQSAPESEY TARI.F.SDEK LVRAKIMEEA ELCTAQTTPQ... ELAPFAA
121  LVGLESLIKQ RIQDAPESEY TRRI.F.NDSA LIDAKIMEEA ELCTAQTTPQ... ELAPFAA
121  PSLEKLIQIL RILGESEY TARI.F.SDEK LVRAKIMEEA ELCTAQTTPQ... ELAPFAA
121  LVQLEQLAE RYKADPTSTY TAKLYASGTR RIAGKVGESE VETALAATVH DRFFLTNEAS
121  LVQLEQLAE RYKADPTSTY TAKLYASGTR RIAGKVGESE VETALAATVH DRFFLTNEAS
121  LVQLEQLAE RYKADPTSTY TAKLYASGTR RIAGKVGESE VETALAATVH DRFFLTNEAS
121  LDFMV*.....
121  L-LE-----
181  223 39.6%
181  DLPYFALTRA V*..... 31.3%
181  DLPYFALTRA V*..... 31.0%
181  DLYVHLVIM NQGGISLEEV RQAKERHQL EGNKEPFTT TAD* 45.4%
181  DLYVHLVIL QDQDLDTV IENLRKHQ* 39.1%
181  DLYVHLVIL QDQDLDTV IENLRKHQ* 40.9%
181  DLYVHLVIL QDQDLDTV IENLRKHQ* 38.3%
181  Consensus

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Fig. 3. Alignment of the deduced amino acid sequence of the *R. sphaeroides hisI* gene and those of the following micro-organisms: *Methanococcus vannielii* (translated from EMBL data base, accession number X04021), *Neurospora crassa* (Swiss-Prot accession number P07685), *Saccharomyces cerevisiae* (P00815), *Lactococcus lactis* (Q021130), *Shigella flexneri* (P37793), *Salmonella typhimurium* (P10367) and *Escherichia coli* (P06989). Asterisks show the carboxy-terminal extremity of each sequence; # indicates the extremities of partial sequences; dots indicate inserted gaps to maximize the alignment. Highly conserved dipeptides and tripeptides are underlined. The consensus shows amino acids that are conserved in at least seven of the eight compared sequences. Percentages of identity between each amino acid sequence and that of *R. sphaeroides* are also indicated.

present in the databases. ORF1 is preceded by a putative ribosome-binding site (RBS) (Shine & Dalgarno, 1975), located 7 bp upstream of the ATG. The fact that the codon usage of ORF1 is very similar to those of other *Rhodobacter* genes suggests that it really is protein-coding sequence (Bibb *et al.*, 1984).

Owing to its similarity to the *hisI* genes of other micro-organisms, ORF2 was assigned to *hisI*. The *R. sphaeroides hisI* gene is found 33 bp downstream of the ORF1 stop codon and there is a likely RBS, complementary to the *R. sphaeroides* 16S rRNA 3' end (Gibson *et al.*, 1979), located 8 bp upstream of the ATG. No putative promoter sequences or transcription stop signals were identified either upstream or downstream of the *hisI* gene. The *R. sphaeroides hisI* gene is 360 bp long. Comparison with the *E. coli* and *S. typhimurium hisIE* 5'-terminal region (which encodes the cyclohydrolase activity) shows an overall identity of 62.7% and 59.6%, respectively. The derived amino acid sequence is homologous to the N-terminal domain of various HisIE proteins (Fig. 3), showing an overall 62.6% and 64.3% similarity to the HisIE peptides of *E. coli* and *S. typhimurium*, respectively. We therefore conclude that *R. sphaeroides hisI* encodes a unifunctional

protein with a predicted molecular mass of 13.4 kDa, while the bifunctional *E. coli* and *S. typhimurium* proteins have a molecular mass of 22.8 kDa.

The alignment of the sequences of the different gene products, as well as the percentages of identity with the predicted *R. sphaeroides* HisI, are shown in Fig. 3. When conservative substitutions are allowed, similarity is approximately 60%. The similarities are not homogeneously distributed throughout the sequence. Amino acids 50–110 of the *R. sphaeroides* protein show a higher degree of coincidence. As previously described (Beckler & Reeve, 1986), this region is characterized by the conserved presence of three cysteines and three tripeptides (KGE, DCD, SCF), which are probably essential for the function of the protein.

The 1.3 kb fragment from *R. sphaeroides* was able to complement *E. coli* mutants regardless of its orientation in relation to the *lac* promoter of pBluescript SK. This could indicate that *R. sphaeroides* *hisI* was being transcribed in *E. coli* from its own promoter. Nevertheless, a subclone with a 250 bp deletion of the 5' end of the pUA510 insert (Fig. 1b) was unable to complement the mutation although it carried an intact *hisI* gene, indicating that this region contains the promoter from which the transcription of the gene occurs. The analysis of the sequence demonstrated that the transcription of *hisI* in pUA510 might start at the preceding ORF1 promoter: a putative σ^{70} promoter (Lang *et al.*, 1995) is found 28 bp upstream of the ORF1 translational start codon, with the presumptive –10 and –35 regions separated by 18 bp (Fig. 2). The homology with the σ^{70} promoters of *E. coli* would allow recognition by the *E. coli* RNA polymerase.

An *EcoRI*–*HindIII* fragment of 0.7 kb, which contained the whole *hisI* gene and just the 3' end of ORF1, was cleaved from pUA510. Transformation of *E. coli* UA4845 showed that this fragment was able to complement the mutation only when it was cloned in the same orientation as the *lac* promoter from pBSK (Fig. 1b). This demonstrates that ORF1 is unnecessary for the expression of *hisI* in *E. coli*, so long as a promoter in the suitable orientation is provided.

Construction of a *R. sphaeroides* *hisI* mutant

To obtain a *R. sphaeroides* *hisI* mutant by marker exchange, the isolated *hisI* gene was disrupted by insertion of an Ω streptomycin/spectinomycin interposon (Fellay *et al.*, 1987) into an internal *ApaI* site. After several subcloning steps, the mutated *hisI* gene was cloned in the suicide plasmid pSUP202 (Simon *et al.*, 1983). The resulting construct, pUA534, was introduced into a wild-type strain of *R. sphaeroides* by triparental mating. Selection was carried out on Z agar plates containing streptomycin and spectinomycin. Clones that had integrated the whole plasmid by single crossover were discarded by testing for tetracycline sensitivity. The resulting clones were plated on Pfennig plates with and without histidine in order to confirm the loss of the prototrophic phenotype.

The *R. sphaeroides* *hisI* mutant strain UA8123 was also analysed by Southern blotting. A 0.7 kb *EcoRI*–*HindIII*

Fig. 4. Southern analysis of chromosomal DNA of both wild-type (lanes A) and UA8123 (lanes B) *R. sphaeroides*. A *HindIII*–*EcoRI* 0.7 kb probe was used to hybridize with genomic DNA digested with *SmaI* (lanes 1), *SalI* (lanes 2) and *BamHI* (lanes 3). Labelled λ DNA digested with *HindIII* is also shown as a size standard (lane M).

fragment containing *hisI* was used as a probe against total DNA from both wild-type and UA8123 strains. The hybridization bands of the mutant, when digested with either *SalI* or *SmaI*, showed a decreased mobility in relation to the wild-type hybridization pattern, as was expected after the insertion of the 2 kb Ω interposon (Fig. 4). On the other hand, when digested with *BamHI*, which cleaves the Ω interposon, the mutant strain showed two hybridization bands whose summed sizes equalled the size of the wild-type hybridization band.

An additional verification of the UA8123 *hisI* mutant consisted of testing for the recovery of the prototrophic phenotype when complemented *in trans* by the *hisI* gene. To do this, pUA509 was introduced into UA8123. All the resulting transconjugants were able to grow on minimal medium without histidine. These results demonstrate that the mutation caused by the insertion of the Ω interposon is not polar, at least concerning genes of histidine biosynthesis. Fragments *BamHI*–*HindIII* and *BclI*–*HindIII* (Fig. 1b) cloned in pRK404 were also able to complement UA8123. This demonstrates that the *R. sphaeroides* *hisI* is transcribed in this bacterium from an unidentified promoter which is located closer to the start codon. This promoter is not functional in *E. coli*, where the *R. sphaeroides* *hisI* gene is expressed from the previously described ORF1 promoter. Therefore, the main obstacle to the expression of the *R. sphaeroides* *hisI* gene in *E. coli* seems to reside in the recognition of its promoter. *R. sphaeroides* genes are seldom expressed in *E. coli*, because of differences at the transcriptional level, due to the different G+C content (68 mol%, in contrast to the 50 mol% of *E. coli*).

The UA8123 mutant will be very useful for further complementation or transcriptional studies because it

offers a mutated genetic background other than *E. coli*, where, as we have seen, some *Rhodobacter* promoters cannot be expressed.

hisI and *hisE* genes are not physically linked in *R. sphaeroides*

In *E. coli*, and many other micro-organisms, the HisI and HisE functions are encoded in a single gene which gives rise to a bifunctional protein. In *S. cerevisiae* (Bruni *et al.*, 1986), the equivalent to *hisI* (*his4A*) is fused to *his4B* (equivalent to *hisE*) as well as to *his4C* (equivalent to *hisD*, which in turn catalyses two steps of the pathway). In contrast, the *hisI* gene of the archaeobacterium *Methanococcus vannielii* encodes a unifunctional protein and unifunctional *hisE* genes have been found in *Azospirillum brasilense* and *Streptomyces coelicolor*.

The analysis of the *R. sphaeroides hisI* sequence and the absence of polarity of the *hisI::ΩSp/Sm* mutant suggested that *hisI* and *hisE* were not physically linked in this micro-organism. To confirm this hypothesis, plasmid pUA509 was introduced into *S. typhimurium* E311. This strain lacks the HisE function, but not that of HisI. The presence of pUA509 in *S. typhimurium* E311 cells did not abolish their His⁻ phenotype. Moreover, we obtained plasmid pUA556, which contains an 8·8 kb insert, by complementation of UA1701 (*hisE*) with the *R. sphaeroides* genomic library. Different *hisI* mutant strains of *E. coli* and *S. typhimurium* carrying pUA556 were unable to grow in the absence of histidine, suggesting that the *hisI* and *hisE* functions are encoded in independent genes in *R. sphaeroides*, and that these genes lie relatively far apart on the chromosome.

Chromosomal localization of the *R. sphaeroides hisI* gene

To locate the *hisI* gene on the physical map of the *R. sphaeroides* chromosome, total DNA of *R. sphaeroides* was extracted and digested with the low-cutting-frequency enzymes *AseI* and *DraI*. The digestion products were separated by PFGE, transferred to a nylon membrane and hybridized with a 0·7 kb probe containing *hisI*. The *AseI* digestion showed a single hybridization band of 1100 kb, which corresponds to fragment A described by Suwanto & Kaplan (1989a). *DraI* digestions showed a single hybridization band of 660 kb, designated as band C by Suwanto & Kaplan (1989b).

To determine the precise location of the gene in these bands, we took advantage of the fact that the Ω interposon contains *AseI* and *DraI* restriction sites. Therefore, restriction of the DNA from the mutant strain with these enzymes will cause the cleavage of a specific band into two new fragments, which can be directly detected after electrophoresis. Total DNA from both *R. sphaeroides* UA8123 and 2.4.1 was digested with *AseI* and *DraI*. After separation by PFGE, the lane corresponding to the UA8123 *AseI*-digested DNA showed two bands of 420 ± 20 and 680 ± 20 kb in size, which did not appear in the lane corresponding to the wild-type strain, while the

Fig. 5. Physical location of the *R. sphaeroides hisI* gene (in bold in chromosome I, deduced from the sizes of the hybridization bands obtained after pulsed-field gel electrophoresis of chromosomal DNA from both wild-type and UA8123 *R. sphaeroides*, digested with *AseI* and *DraI*. Cleavage map for *AseI* (outer circle) and *DraI* (inner circle), map co-ordinates and some previously reported genes are indicated as a reference (Mackenzie *et al.*, 1995).

1100 kb band had disappeared. On the other hand, the UA8123 *DraI*-digested DNA produced two bands, of 230 ± 10 and 430 ± 10 kb in size, instead of the 660 kb band of the wild-type. These results were confirmed by hybridization using the *EcoRI*–*HindIII hisI* probe.

Taking these data into account, the *R. sphaeroides hisI* gene could only be located at co-ordinate 2275 ± 20 kb of chromosome I, that is, about 420 kb from the *AseI* A–K and *DraI* C–D junctions (Fig. 5), according to the *R. sphaeroides* map co-ordinates described by Mackenzie *et al.* (1995).

Finally, to confirm that *hisI* and *hisE* are not physically linked, a DNA fragment from pUA556 was used as a probe to hybridize with *AseI* and *DraI* digests of *R. sphaeroides* 2.4.1 genomic DNA. The probe hybridized with the *AseI* A (1100 kb) and the *DraI* M (55 kb) bands (Mackenzie *et al.*, 1995). This confirms that there are about 300 kb between the *hisI* and *hisE* genes of *R. sphaeroides*, and also that the histidine biosynthesis genes in this bacterium are not encoded in a single transcriptional unit like that of *Enterobacteriaceae*. In agreement with this, the *R. sphaeroides hisA* and *hisB* genes have been determined to be located approximately at map co-ordinates 1900 and 2350 of chromosome I, respectively (Mackenzie *et al.*, 1995).

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