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Cloning and functional characterization of the genes encoding 3-dehydroquinate synthase (*aro*B) and tRNA-guanine transglycosylase (*tgt*) from *Helicobacter pylori*

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Abstract The *aro*B gene from *Helicobacter pylori* strain P1 was cloned and further characterized by sequence analysis and by functional complementation of the *aro*B mutation in *Escherichia coli*. The *aro*B gene encodes the enzyme 3-dehydroquinate synthase which catalyzes one of the early steps in the shikimate pathway. This pathway, which creates aromatic molecules from sugar precursors, is present in prokaryotes, fungi and plants but is absent from mammalian cells. The predicted amino acid sequence of the *H. pylori aro*B gene product showed significant homology (30–40% identity and 50–60% similarity) to 3-dehydroquinate synthases from various other prokaryotes and eukaryotes. The single gene on a plasmid was biologically active in *E. coli*. It suppressed the specific phenotype of *aro*B mutants by restoring the shikimate pathway-dependent synthesis of aromatic amino acids and the production of the siderophore enterobactin. Two other reading frames were found adjacent to the *aro*B gene. The first, designated as *orf*1, had no significant homology to proteins and genes present in databases, whereas the second was found to share a significant degree of homology with the *tgt* gene encoding tRNA-guanine transglycosylase from a variety of other bacteria (40–50% identity and 60–70% similarity). The function of the *tgt* gene was confirmed by heterologous complementation. The gene on a plasmid was shown to complement the queuosine biosynthesis defect in a genetically defined *tgt*$^-$ strain of *E. coli*. The presence of the *aro*B gene and the putative *tgt* homologue in unrelated *H. pylori* strains was confirmed by Southern blot hybridization and by polymerase chain reaction with specific primers.

Key words Shikimate pathway · Iron uptake · Queuosine synthesis · tRNA modification

This work was dedicated to Dr Wolfgang Bredt on the occasion of his 60th birthday in August 1997 to express our gratitude for his continuous support and encouragement

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Introduction

Helicobacter pylori is a gramnegative, microaerophilic, spiral-shaped bacterium that colonizes the human stomach [7]. It causes a chronic inflammatory response leading to active, chronic superficial gastritis and, in some cases, to peptic ulceration [27] and to gastric neoplasia [1]. The pathogen occupies a position beneath the mucus layer in close contact with gastric epithelial cells. After establishment of the infection, *H. pylori* can persist for decades. During the search for a vaccine, considerable effort has been directed towards the identification and cloning of individual *H. pylori* antigens that are effectively recognized by the immune system [29, 30, 37, 51]. Construction of mutant strains of attenuated virulence is an alternative approach for vaccine development.

The shikimate biosynthesis pathway for generation of aromatic compounds from sugar precursors is present in bacteria, fungi and plants, but is absent from mammalian cells ([40], reviewed in [39]). In bacteria, aromatic amino acids and para-amino benzoic acid (PAB) are synthesized from the common precursor chorismate, which is the product of the shikimate pathway. PAB is the precursor for folic acid synthesis, a substance that is not produced by chordates. In consequence, mutants of pathogenic bacteria, which lack any of the enzymes of the shikimate pathway, require aromatic amino acids and PAB for growth, and are thus unable to grow in the host. It has been shown earlier

that mutants of *Salmonella* ([12, 17], reviewed in [10]), *Shigella* [33], and *Yersinia* [36] carrying defects in shikimate pathway genes, were attenuated in vivo, and of potential use as live vaccines.

We report here the cloning and functional characterization of the *aro*B gene of *H. pylori* that encodes the shikimate pathway enzyme 3-dehydroquinate (DHQ) synthase [EC 4.6.1.3]. The shikimate pathway of bacteria, including the function of the AroB protein, has been extensively studied in *Escherichia coli* [19, 31, 38–39]. The enzyme catalyzes the formation of 3-dehydroquinate by cyclization of the sugar precursor 3-deoxy-D-arabino-heptulosonate-7-phosphate. The reaction, which depends on NAD^+ as a cofactor, represents the second step in the shikimate pathway.

In *E. coli* and other enteric bacteria the shikimate pathway is linked to iron metabolism as the synthesis of the siderophore enterobactin depends on chorismic acid from the shikimate pathway (reviewed in [16]). In consequence *aro*B mutants of *E. coli* fail to grow in minimal media and cannot survive iron deprivation.

A reading frame which encodes the Tgt protein was found in some distance to *aro*B. The *tgt* gene encodes the tRNA modification protein tRNA-guanine transglycosylase [EC 2.4.2.29] an enzyme which uses zinc as cofactor [11]. The Tgt protein modifies specifically $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{Tyr}$, and $tRNA^{His}$ by exchange of the genetically encoded guanine in the anticodon with the precursor 7-aminomethyl-7-deazaguanine (preQ) of the hypermodified tRNA nucleoside queuosine [Q: 7-(((4,5-cis-dihydroxy-2-cyclopentene-1-yl)amino)methyl)-7-deazaguanosine)))].

The function of the Q-modification of tRNA is still unknown. Homologues of the *tgt* gene were detected in a variety of species including *E. coli* [42], *Shigella flexneri* [15], *Haemophilus influenzae* [18], and *Zymomonas mobilis* [41]. The three-dimensional structure of the Tgt protein from *Z. mobilis* was recently studied in detail and amino acid residues involved in the enzymatic function of the enzyme and in the binding of the zinc cofactor were inquired [11, 44, 45]. These amino acids were striktly conserved in the deduced Tgt protein from *H. pylori.*

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used are listed in Table 1. The *H. pylori* strains were grown in Brucella broth supplemented with 5% fetal calf serum. The cultures were incubated at 37 °C in a microaerobic atmosphere (85% nitrogen, 10% carbon dioxide, 5% oxygen). *E. coli* strains carrying recombinant DNA on plasmids pBluescript KS^+ and pSO50 were grown in Luria-Bertani (LB) medium [32] supplemented with ampicillin (100 mg/l) and tetracycline (20 mg/l) respectively. Recombinant plasmids carrying *H. pylori* DNA were propagated in the *E. coli* strain HB101.

Growth under iron restricted conditions was monitored on bacto nutrient broth agar (NB, DIFCO) supplemented with 200 μM of the iron chelator 2′,2′-dipyridyl (Sigma). Auxotrophy for the synthesis of aromatic amino acids was assessed by growth on minimmal medium M9 [32].

Table 1 Relevant characteristics of bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Helicobacter pylori		
G27	wt, *cag*A^+, *vac*A (s1b/m1)[a]	A. Covacci
ATCC 43504	wt, *cag*A^+, *vac*A (s1a/m1)[a] identical with NCTC 11637	ATCC
P1	wt, *cag*A^+, *vac*A (s1a/m1)[a]	[35]
NCTC 11638	wt, *cag*A^+, *vac*A (s1a/m1)[a]	[8]
151	wt, *cag*A^+, *vac*A (s1b/m1)[a]	M. Kist
Escherichia coli		
E181	HB101 carrying λCH616 prophage	[35]
HB101	F^-, *rec*A13, *ara*-14, *pro*A2, *leu*B, *lac*Y1, *gal*K2, *rps*L20(str^r), *xyl*-5, *mtl*-1, *sup*E44, *thi*-1, *hsd*S20 (r_B, m_B)	[6]
H1443	*aro*B, *ara*D, *lac*, *rps*L, *thi*	[52]
SJ1505	*ara*D139Δ (*arg*F-*lac*) U169 *thi deo*C *rel*A1 *rps*L150 *tgt*1	[41]
Plasmids		
pBluescripts KS^+	Plasmid cloning vector, Ap^r	Stratagene
pTn*Max*5	cat_{GC}, *res*, *ori*fd, *tnp*R, *tnp*A, *lac*I^q, Cm^r	[25]
pSO50	ori_{colEI}, *ori*T, t_{fd}, P_{iga}, (derivative of pMIN2, described in [25] and [35]), Tc^r	R. Haas
pARO1	pSO50 with a 4 kb chromosomal DNA fragment carrying the genes *aro*B *and tgt* from *H. pylori* P1, Tc^r	This study
pARO1.1	pARO1 with Tn*Max*5 inserted into the *aro*B gene, Cm^r, Tc^r	This study
pARO1.2	pARO1 with Tn*Max*5 inserted into the *tgt* gene, Cm^r, Tc^r	This study
pARO2	pBluescript containing a 1.5-kb *Eco*RI/*Hin*dIII fragment from pARO1 carrying the *aro*B gene, Ap^r	This study
pARO3	pBluescript containing a 2.5-kb *Eco*RV/*Sal*I fragment from pARO1 carrying the *tgt* homologue, Ap^r	This study

[a] The *cag*A status and the *vac*A allele type of the *H. pylori* strains were determined as previously described [2]

DNA: isolation and cloning procedures

Total genomic DNA was isolated from *H. pylori* strains, previously grown in liquid culture, by repeated treatment with phenol and chloroform followed by ethanol precipitation according to standard methods [46]. Plasmid-DNA was isolated from *E. coli* liquid cultures by anion exchange chromatography using a kit (plasmid midi kit, Qiagen). All steps were performed according to a protocol given by the manufacturer.

The plasmid pARO1, which carries the *H. pylori aro*B and *tgt* genes on a 4-kb DNA fragment, was isolated from a plasmid-based DNA library of strain P1 cloned in plasmid pSO50 a derivative of the minimal vector pMin1 [25]. The library was established as previously described for an equivalent library in plasmid pMin2 [35]. The subcloning of defined DNA fragments in pBluescript KS^+ was done according to standard methods [46].

Transposon mutagenesis of pARO1

Transposon insertion mutagenesis of the DNA region cloned in pARO1 was performed using Tn*Max*5 [25], according to a procedure previously described [35]. The plasmid pARO1 was introduced into *E. coli* E181 harbouring plasmid pTn*Max*5 and the transformants were grown in 100 ml LB supplemented with chloramphenicol (20 mg/l), tetracycline (20 mg/l) and 100 μM IPTG to induce transposition. Plasmids in which the *aro*B gene was inactivated by insertion of Tn*Max*5, were selected after transfer into *E. coli* H1443 by their inability to restore growth of the mutant under conditions of iron restriction. The plasmids were isolated and the insertion sites of the transposon were mapped by restriction with enzymes *Eco*RI and *Hin*dIII. The plasmids pARO1.1 and pARO1.2 which carry a single insertion of Tn*Max*5 in the *aro*B gene and in the *tgt* gene, respectively, were chosen for further analysis.

Complementation of the *tgt* mutation

Biochemical complementation of the *tgt* mutation was assayed as described elsewhere [41] using the *E. coli* strain SJ1505.

DNA sequence determination

Sequencing was performed by primer walking according to a modification of the dideoxynucleotide chain-termination method [47]. The nucleotide sequence was determined on both strands. The plasmids pARO2 and pARO3 were used as templates for the sequencing reactions which were done with a PRISM Ready Reaction Dye Cycle Sequencing kit (Applied Biosystems) including fluorescence-labeled dideoxynucleotides. The fluorescent reaction products were separated on a denaturing polyacrylamide gel and analyzed by an Applied Biosystems 373 A automated DNA sequencig machine. The DNA sequence was analyzed with the HUSAR software package provided by the German Cancer Research Center in Heidelberg. The detailed alignments were done using GAP, CLUSTAL, or BESTFIT.

Conditions for PCR amplification

PCR products carrying parts of the *aro*B and *tgt* regions were generated using primers F (CTAAAAACAAGCGTTCG), F1 (TGGACAATAGCTCTTGG), together with R (CGTGATAGAGTCCGGAG), R1 (ACACGCGCTTTTGGTG), and with F2 (CTTTTAGATTTAGCCCA) together with R2 (AAGCTGGCTAAACGAG), respectively. The locations of the primers are given in Fig. 1. The reactions were carried out with 100 ng template DNA, 25 pmoles of each primer, 1 Unit *Taq* polymerase (Pharmacia) in 50 μl of a PCR buffer system that has been described previously [3]. Denaturation, annealing, and polymerization steps were done for 1 min each at 93°, 50°, and 72 °C, respectively. After an initial denaturation for 2 min at 94 °C the cycles were repeated 25 times followed by a final polymerization step at 72 °C for 10 min.

Southern blot hybridization

Southern blot hybridization [48] for detection of *aro*B and *tgt* was performed using the digoxygenin DNA labeling and detection kit from Boehringer-Mannheim, according to a protocol given by the manufacturer. To generate probes, specific for *aro*B and *tgt*, internal fragments of both genes present on plasmid pARO1 were amplified by PCR with primers indicated in Fig. 1. The PCR products were labeled with digoxygenine-11-dUTP by nick-translation. Isolated DNA (20 μg) from various *H. pylori* strains was completely fragmented with *Hin*dIII and separated on a 1% agarose gel. After depurination, denaturation and neutralization, the DNA fragments were transferred to a positively charged nylon membrane (Quiabrane plus) by capillary forces and cross-linked for 2 min by UV irradiation (203 nm). The following steps, including hybridization, washing and detection of the probe, were done according to the manufacturers recommendations. Hybridization was done at 57 °C for 16 h in a buffer without formamide and stringency washing was carried out at 65 °C.

Nucleotide sequence accession number

The complete DNA sequence of the fragment cloned in pARO1 has been assigned EMBL database accession number Y12061.

Results

Isolation of a DNA fragment carrying the *aro*B gene from *Helicobacter pylori*

As part of a project focussed on characterization of iron uptake determinants in *H. pylori*, we screened a plasmid based library of *H. pylori* strain P1 for restoration of growth of the *E. coli aro*B mutant strain H1443 under iron-restricted conditions. Due to the lack of siderophore synthesis, which depends on the shikimate biosynthesis pathway, growth of the mutant H1443 is blocked by the defect in *aro*B. Thus, it is unable to grow under iron-limited conditions in NB agar with addition of 200 μM of the iron chelator 2′,2′-dipyridyl. A plasmid based gene library containing DNA fragments from *H. pylori* strain P1 in plasmid pSO50 was isolated from *E. coli* HB101 and introduced into H1443 by transformation. Some plasmids which carried DNA fragments from *H. pylori* restored iron uptake, resulting in growth on NB agar containing dipyridyl. From 13 transformants which grew, plasmids were isolated and subjected to restriction analysis with various enzymes (Fig. 1). The fragment patterns obtained after gel electrophoresis indicated that all plasmids carried an identical DNA fragment of 4 kb in size. This plasmid was designated as pARO1 and the DNA region which was responsible for the complementation was further investigated by transposon mutagenesis and by subcloning. The 1.5-kb *Eco*RI/*Hin*dIII and the 2.5-kb *Eco*RV/*Sal*I fragment derived from pARO1 were separately cloned in plasmid pBluescript and the resulting plasmids were designated as pARO2 and pARO3, respectively. The plasmid pARO1 was mutagenized with transposon Tn*Max*5 as described in the methods section and all transposon insertions that abolished the complementation of H1443 were exclusively located within the 1.5-kb *Eco*RI/*Hin*dIII fragment of the

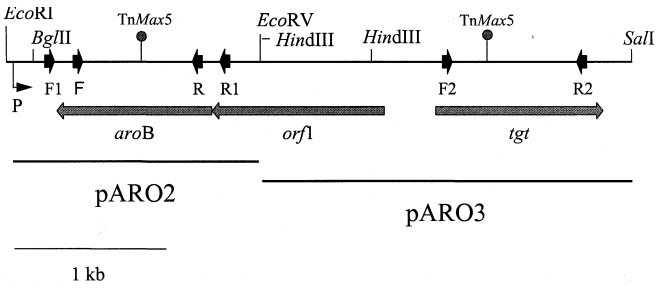

Fig. 1 Restriction map of the *aro*B region clones from *Helicobacter pylori* strain P1. The *upper line* represents the DNA region cloned in pARO1. The DNA fragments subcloned into pARO2 and pARO3 are *underlined.* The restriction sites for *Eco*RI and *Sal*I are part of the cloning vector pSO50. The *gray arrows* represent reading frames for genes *aro*B, *tgt* and for the putative open reading frame *orf*1. The *circles* indicates insertion sites of Tn*Max*5 in plasmids pARO1.1 and pARO1.2 respectively. The location and orientation of the binding sites for oligonucleotide primers F, F1, F2 and R, R1, R2 are marked by the *black arrowheads.* The location and orientation of the gonococcal IgA protease promoter is also marked (*P*)

4-kb fragment in pARO1. Plasmids pARO1.1 and pARO1.2 which carry insertions within the middle of the fragments subcloned in pARO2 and pARO3, respectively, were chosen for further analysis. To confirm the results obtained from transposon mutagenesis, plasmids pARO1.1, pARO1.2, pARO2 and pARO3 were isolated and reintroduced into the mutant H1443 and the growth of the transformants under iron-restricted conditions was monitored. The results summarized in Table 2 indicate that the 1.5-kb fragment cloned in pARO2 was sufficient for complementation of the *aro*B mutation, whereas plasmid pARO3 did not restore growth under iron depletion. The transposon insertion in pARO1.1 abolished growth whereas plasmid pARO1.2 which carried Tn*Max*5 in the larger *Eco*RV/*Sal*I fragment was still able to complement the *aro*B mutation. To investigate whether the 1.5-kb DNA fragment cloned in pARO2 could bear an *aro*B homologue of *H. pylori* rather than an iron uptake system, growth of H1443 (pARO2) was also monitored on minimal medium M9. Growth of the mutant on M9 medium strictly depends on the AroB function, since it restores the synthesis of aromatic amino acids which are not present in the medium. The fact that pARO2 but not pARO3 restored growth of H1443 on M9 medium (Table 2) confirmed that the 1.5-kb DNA fragment contains the *aro*B gene of *H. pylori* which is biologically active in *E. coli.*

Table 2 Complementation analysis of the *aro*B gene from *H. pylori* (*NB-DP* nutrient both containing 200 μM 2′,2′-dipyridyl, *M9* minimal medium without addition of aromatic amino acids, + growth, – no growth, *nd* not determined)

Strain	Relevant genotype	Plasmid	Plasmid-genotype	Growth on	
				NB-DP	M9
H1443	*aro*B	None	–	–	–
		pBluescript	–	–	–
		pARO1	*aro*B, *tgt*	+	+
		pARO1.1	*aro*B::Tn*Max*5	–	nd
		pARO1.2	*tgt*::Tn*Max*5	+	nd
		pARO2	*aro*B	+	+
		pARO3	*tgt*	–	–

Sequence analysis of the *aro*B gene

To identify and to further characterize the *aro*B gene of *H. pylori*, the 1.5-kb DNA fragment cloned in pARO2 was sequenced on both strands. Computer-assisted analysis of the nucleotide sequence identified the reading frame for *aro*B (Fig. 2 A), which was seen to be 50% identical to the *aro*B gene of *E. coli*. The 1032 nucleotides (nt) code for a predicted protein with a calculated molecular mass of 39 245 Da. The DNA region in front of the ATG start codon contains a putative ribosome binding site at position –12 (CGGA) and various DNA motifs with homology to σ^{70}-promoters of *E. coli*, which could represent possible binding sites for RNA polymerase. The alignment of the predicted amino acid sequence of the *H. pylori* AroB protein with the sequences of 3-DHQ synthases from other bacterial species revealed a significant degree of homology (Fig. 3 A). The *H. pylori* protein was found to be 30 – 40% identical and 50 – 60% similar to other bacterial AroB proteins. The degree of homology did not vary between gram-positive and gram-negative bacteria and the *H. pylori* protein was 37%, 39%, 38%, and 33% identical with AroB from *E. coli*, *H. influenzae*, *Bacillus subtilis*, and *Mycobacterium tuberculosis*, respectively. The alignment to the multifunctional AroM protein of yeast and fungi in which the AroB function is localized within the first 392 amino acids, exhibited a similar degree of homology (30% identity and 50% similarity). The mid part of the AroB protein is more conserved than the N terminus or the C terminus, which show both a high degree of variability. Due to a deletion within the N terminus the amino acid sequence of the *H. pylori* AroB is 19 amino acids shorter than the corresponding AroB proteins from *E. coli*, *B. subtilis*, *H. influenzae*, and *M. tuberculosis*, which share an identical size of 362 amino acids.

Identification, sequence analysis and functional characterization of the *tgt* gene

The DNA fragment subcloned in plasmid pARO3 was completely sequenced and computer analysis revealed the presence of two additional open reading frames (Fig. 1). The amino acid sequence deduced from the first reading frame *orf*1 which codes for a putative protein of 383 amino acids showed no homology to proteins present in databases. The second open reading frame encoding 371 amino acids was named *tgt*, because it shared significant homology 40 – 50% identity and 60 – 70% similarity) with bacterial tRNA-guanine transglycosylase (Tgt) proteins present in databases. The 1113 nucleotides code for a predicted protein with a calculated molecular mass of 41 300 Da (Fig. 2 B). The DNA region in front of the ATG start codon contains a putative ribosome binding site at position –8 (AAGG) and

possible binding sites for RNA polymerase with weak homology to the σ^{70}-dependent promoter of *E. coli*. A more detailed alignment of the deduced amino acid sequence (Fig. 3 B) revealed that the predicted Tgt protein is 45.9%, 46.4%, 44.7%, and 43.9% identical with Tgt from *E. coli*, *S. flexneri*, *H. influenzae*, and *Z. mobilis*, respectively.

The Tgt proteins from *E. coli* and *Z. mobilis* were recently investigated by site directed mutagenesis [11, 43, 45] and by crystal structure analysis [44]. Functionally important residues involved in the coordination of the zinc cofactor, in the binding of the preQ substrate, and in the catalytic activity are strictly conserved within Tgt proteins from various prokaryotes including Tgt from *H. pylori* (Fig. 3 B).

The function of the *H. pylori tgt* gene was further investigated by heterologous complementation of the *tgt* mutation in *E. coli*. Therefore, pARO3 was introduced into the *tgt*$^-$ mutant strain *E. coli* SJ1505. As assessed by an assay described previously [41] the plasmid lead to restoration of queuosine synthesis in this strain, indicating the tRNA-guanine transglycosylase function of the *tgt* gene product.

Detection of *aro*B and *tgt* by hybridization and by PCR analysis

The presence of the *aro*B and *tgt* genes in various strains of *H. pylori* was confirmed by Southern blot hybridization (Fig. 4). One single fragment hybridized in the *Hin*dIII-digested genomic DNA of strains G27, NCTC 11638, P1, ATCC 43504, and 151 when analyzed with digoxygenin-labeled probes carrying sequences from *tgt* and *aro*B. The size of the fragments varied within strains indicating restriction fragment length polymorphisms (RFLPs). With the *aro*B gene as probe, a 2.7-kb DNA fragment was detected in strains NCTC 11638, P1 and ATCC 43504, whereas in DNA of strains G27 and 151 the probe hybridized to smaller fragments of 1.7 and 2 kb, respectively (Fig. 4). The *tgt* gene hybridized to fragments which were 2.5 kb (strain G27), 3.6 kb (strains P1, 151) and 5.1 kb/ (strains NCTC 11638, ATCC 43504) in size (not shown).

The genomic DNA of all strains was furthermore analyzed by PCR with primer pairs F/R, F1/R1, F2/R2, which amplify parts of the coding regions of *aro*B and of *tgt*, respectively. The identical size of PCR products amplified from DNA of all five strains indicated that the RFLPs seen in Southern blot hybridization experiments were rather due to sequence variations in the flanking regions than in the coding regions of both genes (not shown).

Discussion

The isolation of an intact *aro*B gene from *H. pylori* provides evidence for the presence of the shikimate pathway in this organism. Recently, *aro*B and other shikimate pathway genes were identified by homologies within a project focussed on sequencing of the *H. pylori* genome [50]. Furthermore a gene encoding 3-dehydroquinase, an enzyme involved in the catabolization of aromatic molecules, was isolated from *H. pylori* [5]. Both the biosynthetic pathway and the catabolic pathway genes were reported to be present in the human pathogen *M. tuberculosis* [21]. The deduced amino acid sequence of *aro*B from *H. pylori* was found to be similar to 3-DHQ synthases from other bacteria. The degree of homology to AroB from evolutionary-unrelated species like *E. coli*, *B. subtilis*, yeast and fungi was comparable. Within each group the bacterial AroB proteins from *H. influenzae* and from *E. coli* are much more closely related to each other than to the *H. pylori* protein. The same is true for AroB from *S. aureus* and from *B. subtilis* in the other group [34]. It is interesting to note that the predicted *H. pylori* protein shows nearly the same degree of similarity to both prokaryotic and to eukaryotic 3-DHQ synthases. This indicates that the protein was conserved during evolution. Since *H. pylori* is not related to the bacteria from which AroB sequences are already available, its AroB sequence could provide information concerning evolutionary and functional aspects. The mid-part of AroB was more conserved than the N and the C terminus. Therefore, amino acids located in the mid-part of the sequence could be of functional importance. This assumption is underlined by the fact that a consensus sequence within amino acids 96–126 proposed for NAD^+ binding in *E. coli* [31] are conserved within AroB proteins from *H. pylori* and from other species.

The *aro*B gene of *H. pylori* was found to be biologically active in *E. coli*. It suppressed the specific phenotype of *aro*B mutants demonstrating the 3-DHQ synthase activity of the coded protein. The expression of a functional *H. pylori* AroB protein in *E. coli* was surprising, because the enzyme catalyzes a relatively complex biochemical reaction and the overall homology between proteins from both species is relatively low. On the other hand it has been previously shown that the genes from either gram-positive and gram-negative bacteria can complement the mutation in *E. coli* [21, 34]. The expression of the *H. pylori* AroB protein in *E. coli* depends on correct transcription. Functional complementation of *E. coli aro*B mutants was observed independently from the orientation of the *aro*B gene with respect to promoters and reading frames of the vector, indicating that the promoter of *H. pylori aro*B might be active in *E. coli*.

The genetic organization of the shikimate pathway genes varies among different bacterial species. In *E. coli* [19, 31], and in other enteric bacteria, the *aro* genes are located on distinct regions of the chromosome. The *aro*A gene is part of an operon that contains *ser*C [13] and the *aro*B gene is located near the *aro*K gene in the *dam* operon [28]. In *B. subtilis* the organization of *aro* genes is completely different, the genes are part of a large cluster responsible for synthesis and transport of aromatic compounds [22]. The *aro*B genes of various other prokaryotes like *H. influenzae* [18], *M. tuberculosis* [21], and *S. aureus* [34] has been isolated and it seems to be a general principle that in prokaryotes shikimate pathway enzymes are coded by separate genes. In contrast, eukaryotes like *Saccharomyces cerevisiae* [14] or *Aspergillus nidulans* [9] carry the pentafunctional AroM protein which coordinates the activities of all shikimate pathway enzymes.

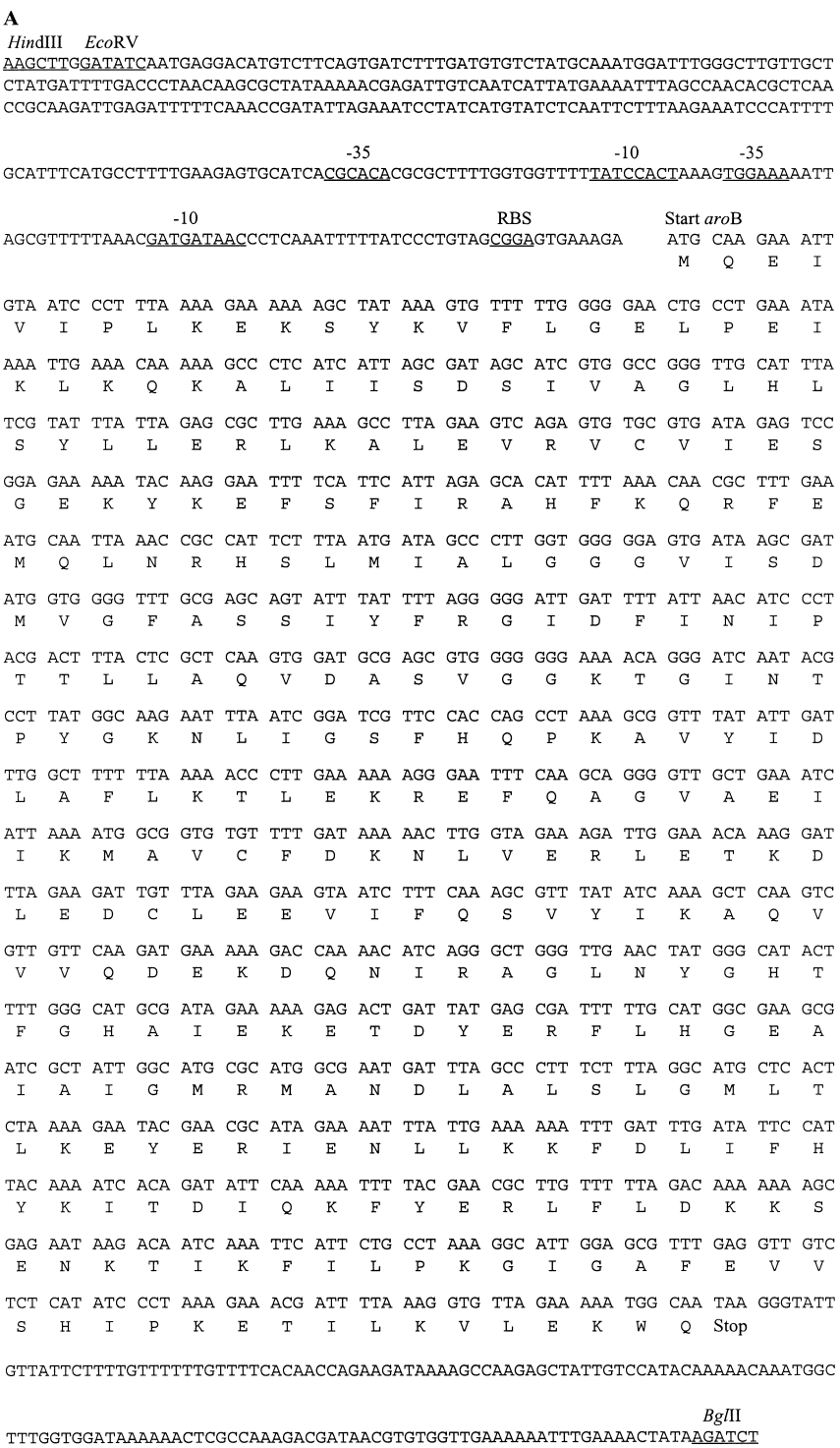

Fig. 2 A, B DNA sequence of the *H. pylori aro*B and *tgt* genes. The coding regions of the *aro*B gene (**A**) and of the *tgt* gene (**B**) are translated. Possible consensus structures with homologies to σ^{70}-dependent promoters (–10 and –35) of *Escherichia coli* and the putative ribosome binding sites (*RBS*) are *underlined.* Restriction sites for enzymes *Hin*dIII, *Eco*RV and *Bgl*II are also *underlined*

The putative gene *orf*1 which is located upstream from *aro*B with respect to the direction of transcription showed no significant homology to genes and proteins present in databases including enzymes involves in the shikimate pathway. This indicates that the shikimate pathway genes are separately located on the *H. pylori* chromosome as confirmed by the results of the *H. pylori* sequencing project, in which the genes coded on pARO1 were found to be in the same arrangement [50]. The possible function of the *orf*1 gene is under investigation.

The *tgt* gene was identified by the significant homology of the encoded protein to equivalent enzymes from other bacterial species. The fact that all amino acids known to be essential for the function of the Tgt protein from *Z. mobilis* are conserved in Tgt from *H. pylori*, provided strong evidence that the gene product has a catalytic activ-

B

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                                    -35             -10
CACCAAGTCTAAAAAATTTTTTGCTACAATGCCATCTAAAATAAGGGCGATTTTTTTCAATTCCAAACTTCCTAA
                                        RBS             Start tgt
AAAATGATTACGCCATTATATCCAAGATTAAGGCTTAAACG   ATG GAT TTT CAA CTC CAA GCG ACT
                                             M   D   F   Q   L   Q   A   T

GAC AAA CAC GCG CGA GCT GGT CTT TTA GAT TTA GCC CAT TCT CAA GTG GCA ACG CCT
 D   K   H   A   R   A   G   L   L   D   L   A   H   S   Q   V   A   T   P

GTT TTT ATG CCC GTA GGC ACG CAA GGC TGC ATC AAA TCT TTA GAC GCT ACA GAT GTG
 V   F   M   P   V   G   T   Q   G   C   I   K   S   L   D   A   T   D   V

CAA GGA ATT TTA GGC GCT AAA CTC ATT TTA GCC AAC ACC TAT CAC ATG TAC TTA AGG
 Q   G   I   L   G   A   K   L   I   L   A   N   T   Y   H   M   Y   L   R

CCG GGT GAG AAA GTG GTT GGA CAA TTA GGG GTC TTG CAT CAT TTC GCT CAA TTT CAG
 P   G   E   K   V   V   G   Q   L   G   V   L   H   H   F   A   Q   F   Q

GGG AGT TTT TTA ACC GAT AGT GGA GGG TTT CAA GCC TTT AGC TTG AGC GAT AAT GTC
 G   S   F   L   T   D   S   G   G   F   Q   A   F   S   L   S   D   N   V

AAA TTG CAA GAA GGC GGG ATT GTT TTT AAA TCC CAT ATT GAT GGG AAC AAT CAT TTA
 K   L   Q   E   G   G   I   V   F   K   S   H   I   D   G   N   N   H   L

TTC ACG CCC GCT AAA GTT TTG GAC ATT CAA TAT TCT TTA AAT AGC GAT ATT ATG ATG
 F   T   P   A   K   V   L   D   I   Q   Y   S   L   N   S   D   I   M   M

GTT TTA GAC GAT TTA GTG GGC TTG CCC GCT CCC TTA AAA CGC CTT GAA GAA TCC ATT
 V   L   D   D   L   V   G   L   P   A   P   L   K   R   L   E   E   S   I

AAA AGA AGC GCT AAA TGG GCG AAT CTC AGC CTA GAA TAC CAC AAA GAA AAT AAC CGC
 K   R   S   A   K   W   A   N   L   S   L   E   Y   H   K   E   N   N   R

CCC AAC AAC AAC CTT TTT GCC ATT ATC CAG GGC GGC ACG CAT TTG AAA ATG CGC AGT
 P   N   N   N   L   F   A   I   I   Q   G   G   T   H   L   K   M   R   S

CTT AGC GTG GGA TTG ACG CAT AAG GGT TTT GAT GGC TAT GCT ATA GGC GGT TTA GCG
 L   S   V   G   L   T   H   K   G   F   D   G   Y   A   I   G   G   L   A

GTG GGG GAA AGC GCT GAT GAA ATG CTA GAA ACC ATC GCG CAC ACC ACC CCC TTG CTC
 V   G   E   S   A   D   E   M   L   E   T   I   A   H   T   T   P   L   L

CCT AAA GAC AAG CCT CGC TAC CTA ATG GGC GTA GGC ACG CCT GAA AAT ATC CTA GAC
 P   K   D   K   P   R   Y   L   M   G   V   G   T   P   E   N   I   L   D

GCT ATC GGT TTA GGG GTG GAT ATG TTT GAT TGC GTG ATG CCC ACC AGA AAC GCC AGA
 A   I   G   L   G   V   D   M   F   D   C   V   M   P   T   R   N   A   R

AAC GCC ACC CTT TTC ACG CAT TCT GGC AAA ATT TCT ATC AAA AAC GCG CCC TAT AAA
 N   A   T   L   F   T   H   S   G   K   I   S   I   K   N   A   P   Y   K

TTA GAT AAT ACC CCT ATT GAA GAA AAT TGT ACA TGT TAT GCT TGC AAA CGC TAT TCT
 L   D   N   T   P   I   E   E   N   C   T   C   Y   A   C   K   R   Y   S

AAA GCC TAT TTG CAC CAT CTC TTT AGA GCT AAA GAA CTC ACT TAC GCT CGT TTA GCC
 K   A   Y   L   H   H   L   F   R   A   K   E   L   T   Y   A   R   L   A

AGC TTG CAC AAT TTG CAT TTT TAT TTA GAG CTG GTT AAA AAC GCC AGA AAC GCC ATT
 S   L   H   N   L   H   F   Y   L   E   L   V   K   N   A   R   N   A   I

TTA GAA AAG CGG TTT TTG AGT TTT AAA AAA GAA TTT TTG GAG AAA TAT AAT TCT CGC
 L   E   K   R   F   L   S   F   K   K   E   F   L   E   K   Y   N   S   R

TCT CAT TGA
 S   H  Stop ATGATGGAATACAAAAATACTAAAAAGCGTTTTTTTCCATCAATGACAAAGAACTTGCAAAGC
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ity very similar to tRNA-guanine transglycosylase. This was confirmed by heterologous complementation of the *tgt* mutation of *E. coli.* The presence of the *tgt* gene in *H. pylori* is a strong indication that tRNA modification by queuosine (Q) might exist in the bacterium. This specific modification of tRNA takes place in eukaryotes and in prokaryotes with the only difference being that Q is a nutrient factor for eukaryotes, whereas bacteria synthesize Q de novo. In consequence, eukaryotes contain Q as the free base, whereas in prokaryotes Q is always a component of tRNA. Bacteria synthesize preQ from GTP as pteridines and riboflavin and genes encoding enzymes involved in this biosynthetic pathway were isolated from *E. coli* [42].

The function of the tRNA modification catalyzed by Tgt is not well understood. There is evidence that in eukaryotes variations in this specific tRNA modification might be in-

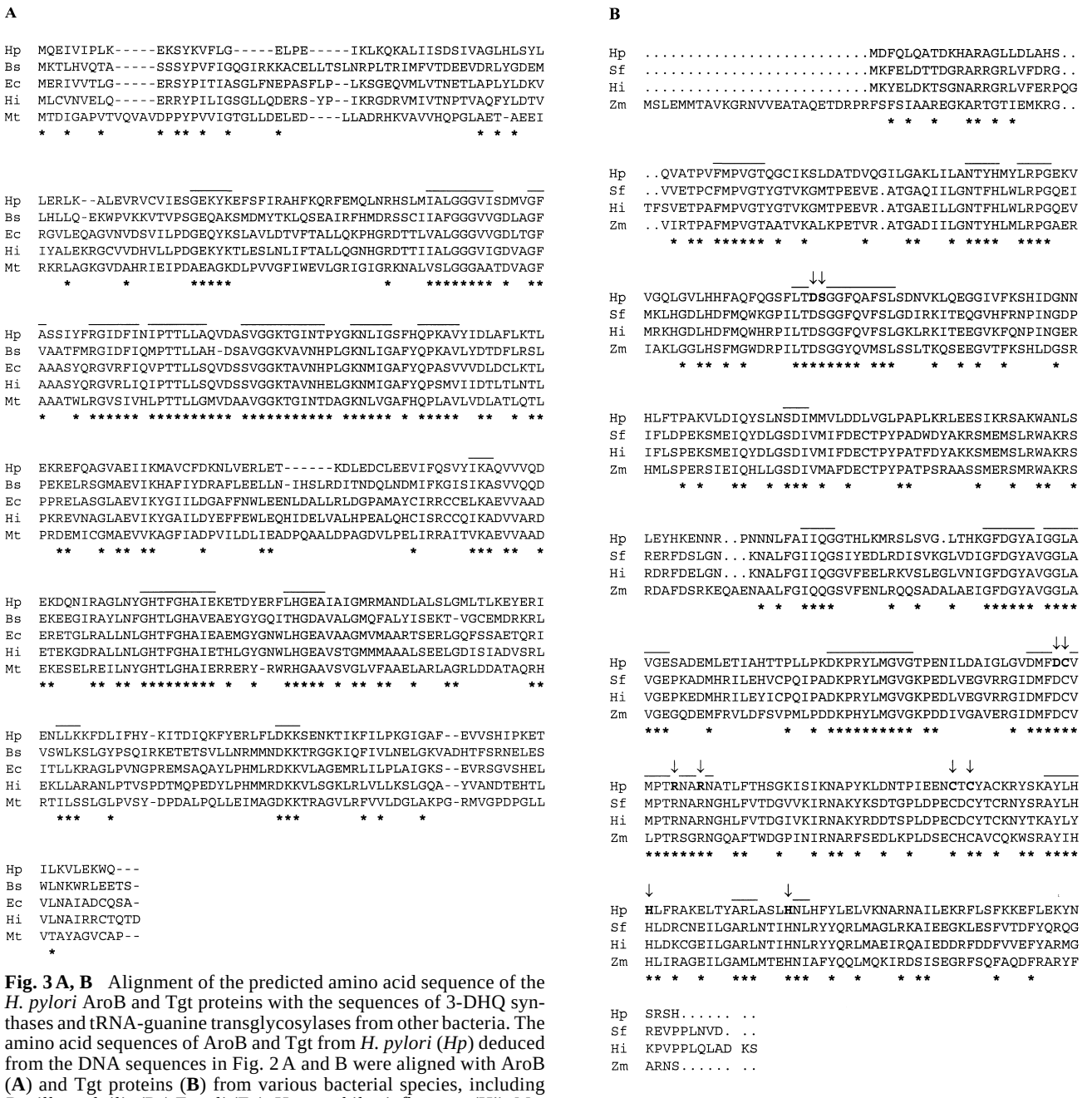

Fig. 3 A, B Alignment of the predicted amino acid sequence of the *H. pylori* AroB and Tgt proteins with the sequences of 3-DHQ synthases and tRNA-guanine transglycosylases from other bacteria. The amino acid sequences of AroB and Tgt from *H. pylori* (*Hp*) deduced from the DNA sequences in Fig. 2 A and B were aligned with AroB (**A**) and Tgt proteins (**B**) from various bacterial species, including *Bacillus subtilis (Bs) E. coli (Ec), Haemophilus influenzae (Hi), Mycobacterium tuberculosis (Mt), Shigella flexneri (Sf),*and *Zymomonas mobilis (Zm)*. The alignment to Tgt from *E. coli* is not shown because it is more than 95% identical with Tgt (VacC) from *S. flexneri*. Amino acids that were found to be identical in proteins from *H. pylori* and in at least two other species, are marked with *asterisks*. Stretches of more than two amino acids that were conserved among a minimum of two species are indicated by *bars*. The *arrows* indicate amino acids which were previously shown to be involved in the function of the Tgt protein of *Z. mobilis* [41]. These were typed in *boldface* when conserved in the sequence of the *H. pylori* protein

volved in various cellular processes (reviewed in [26]) like differentiation and proliferation including cancer development. Accumulation of Q-deficient tRNA was observed, for example, in embryonic tissue and in fast proliferating tumor cells. In prokaryotes tRNA modification by Q is supposed to be involved in the fidelity of translation [41, 42]. Furthermore it has been recently shown that the *tgt* homologue *vac*C of *S. flexneri* is necessary for the expression of proteins essential for invasion of epithelial cells, demonstrating that the Tgt protein can play a role in bacterial virulence.

H. pylori is not closely related to organisms from which molecular data on Tgt and AroB are available so far. Therefore, the regions conserved in both proteins (Fig. 3) might help in further investigation of their enzymatic functions.

Both genes, *aro*B and *tgt*, were detected in various unrelated strains, including type strains and clinical isolates, by Southern blot hybridization using parts of both genes from strain P1 as probe. The probe hybridized to only one restriction fragment in genomic DNA of each strain, suggesting that a single copy of each gene exists per chromosome. Fur-

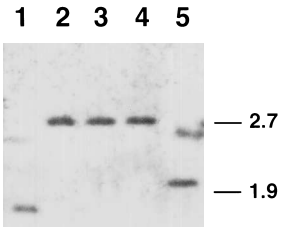

Fig. 4 Detection of the *aro*B and *tgt* genes in DNA from various *H. pylori* strains. The *aro*B gene was detected by Southern hybridization as described in methods. In *lanes 1–5*, 20 µg *Hin*dIII-digested total DNA from strains G27 (*lane 1*), NCTC 11638 (*lane* 2), P1 (*lane* 3), ATCC 43504 (*lane* 4), and 151 (*lane* 5) was separated electrophoretically on a 1% agarosegel. The sizes of marker DNA (in kb) is given on the right

thermore DNA sequence variation was detected among *aro*B and *tgt* genes from different strains. On the other hand, PCR products generated with flanking primers from DNA of all five strains were of identical size, indicating that the primer binding sites were conserved and that length variations detected by hybridization were not due to deletions within the coding regions. It was previously shown that DNA polymorphisms exist for many genes, and sequence variation seems to be a phenomenon common in the *H. pylori* population (reviewed in [49]). In addition to microdiversity of the DNA sequence of single genes, a high degree of macrodiversity exists within the *H. pylori* population, and it was recently shown that the gene order varies among strains [24]. The 4-kb DNA fragment present in pARO1 contained no other reading frames with homology to enzymes of the shikimate pathway. Therefore, it remains unclear if the *aro*B gene of *H. pylori* is organized in a large operon as in *B. subtilis* or if the *aro* genes are unlinked, as shown for enterobacteria.

The synthesis of aromatic molecules is essential for the survival of a variety of bacterial pathogens in the host. Based on this knowledge, it can be suggested that the *aro*B gene might contribute to the virulence of *H. pylori*. The data presented here could provide a tool for construction of *H. pylori aro*B mutants of potential use in vaccine development and for establishing safe strains for bioengineering. The production of siderophores recently described from *H. pylori* [23] raises the question if there is a link between iron acquisition and synthesis of aromatic molecules similar to that seen in *E. coli*. To investigate if mutants which carry defects in the shikimate pathway genes are attenuated in the host, is one of the future goals of ongoing studies.

We have isolated and characterized the *rib*A gene of *H. pylori* which codes for GTP-cyclohydrolase II, an enzyme that catalyzes the first step in the biosynthetic pathway for riboflavin [4]. It has been recently shown, for the animal pathogen *Actinobacillus pleuropneuomiae*, that genetically generated riboflavin auxotrophs are attenuated in the host [20]. It seems reasonable that mutants that carry defects in *rib*A or *aro*B, or in both genes, will also be attenuated in the host. Therefore, the information provided here could be useful for generating attenuated *H. pylori* strains which could be used as safe strains for biological engineering and for the development of live vaccines.

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Note added in proof The amino acid sequences of AroB, Orf1, and Tgt correspond to open reading frames HP0283, HP0282, and HP0281 of the Helicobacter pylori strain S26695 recently published [50], respectively. The sequences of all three proteins were found to be more that 95% identical in both strains (P1 and 26695).