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# Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in Gram-negative bacteria

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**The hemin receptor HemR of *Yersinia enterocolitica* was identified as a 78 kDa iron regulated outer membrane protein. Cells devoid of the HemR receptor as well as cells mutated in the *tonB* gene were unable to take up hemin as an iron source. The hemin uptake operon from *Y.enterocolitica* was cloned in *Escherichia coli* K12 and was shown to encode four proteins: HemP (6.5 kDa), HemR (78 kDa), HemS (42 kDa) and HemT (27 kDa). When expressed in *E.coli hemA aroB*, a plasmid carrying genes for HemP and HemR allowed growth on hemin as a porphyrin source. Presence of genes for HemP, HemR and HemS was necessary to allow *E.coli hemA aroB* cells to use hemin as an iron source. The nucleotide sequence of the *hemR* gene and its promoter region was determined and the amino acid sequence of the HemR receptor deduced. HemR has a signal peptide of 28 amino acids and a typical TonB box at its amino-terminus. Upstream of the first gene in the operon (*hemP*), a well conserved Fur box was found which is in accordance with the iron-regulated expression of HemR.**

*Key words:* hemin/hemin uptake mutant/iron/TonB

## Introduction

Iron restriction encountered in the body fluids of mammals by invading micro-organisms is part of a non-specific defence against unwelcome intruders (Weinberg, 1984). The possession of a highly efficient iron acquisition system is therefore a prerequisite for the successful multiplication of the micro-organism in its host. Some micro-organisms synthesize high affinity iron chelators, siderophores and specific transport systems for the transport of siderophore-$Fe^{3+}$ complexes into the cell interior. The second, energetically less costly strategy of obtaining iron from the host relies on the use of the host's iron chelators such as transferrin, lactoferrin, citrate and heme-containing compounds by highly specific recognition and transport systems (Braun *et al.*, 1991; Otto *et al.*, 1992). An initial step in the majority of these iron assimilation pathways is the recognition and binding of the iron-containing compound to its specific receptor protein located in the outer membrane of the bacterial cell. These receptors are highly selective in binding only their 'own' iron-containing compound and at least in the case of siderophore receptors, they are able to distinguish between iron-loaded and iron-free forms of the siderophore, since the former have a higher affinity for receptor than the latter (Braun and Hantke, 1991). The internalization of the bound siderophores into the periplasmic space depends on the interaction between the loaded receptor and the TonB-ExbBD protein complex located in the cytoplasmic membrane (Braun and Hantke, 1991).

*Yersiniae* can cause clinical pictures ranging from benign diarrhoea to fulminant septic pneumonia. The main virulence determinant of *Yersiniae* is a 70 kb plasmid that carries the *yop* regulon (for review see Cornelis *et al.*, 1989). The role of iron acquisition in the pathogenesis of yersiniosis was made apparent by the recent discovery of two iron regulated proteins present only in highly pathogenic members of *Yersinia* species (Carniel *et al.*, 1989). In addition, production of a siderophore by the more virulent serotypes of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* was demonstrated (Heesemann, 1987). *Y.enterocolitica* serotypes are divided into more virulent, so-called mouse-lethal serotypes (O:8 and O:21) and mouse-nonlethal serotypes (O:3, O:9 and O:5b) (Robins-Browne and Prpic, 1985). Since the main difference between the mouse-lethal and mouse-nonlethal serotypes is the production of the siderophore, iron acquisition by the siderophore-dependent pathway is an important virulence factor of *Y.enterocolitica*. Moreover, if the experimental animals prior to the bacterial challenge are loaded with iron or with the siderophore that *Y.enterocolitica* mouse-nonlethal serotypes can use (Desferal), the course of infection resembles those of mouse-lethal serotypes (Robins-Browne and Prpic, 1985).

Due to their abundance in the host, heme-containing compounds are potentially a valuable source of iron for invading micro-organisms. More than 2 g of heme-iron in hemoglobin (Otto *et al.*, 1992) represents a potentially dangerous reservoir of iron. When it becomes available during hemolysis it makes the host dramatically more susceptible to infections and their complications (Weinberg, 1984). A large number of bacteria use heme compounds as a source of iron including *Haemophilus influenzae* (Coulton and Pang, 1983; Stull, 1987), *Haemophilus ducreyi* (Lee, 1991), *Vibro cholerae* (Stoebner and Payne, 1988), *Vibrio anguillarum* (Mazoy and Lemos, 1991), *Vibrio vulnificus* (Helms *et al.*, 1984), *Neisseria gonorrhoeae* (Dyer *et al.*, 1987), *Plesiomonas shigelloides* (Daskaleros *et al.*, 1991), *Porphyromonas gingivalis* (Gibbons and MacDonald, 1960), *Serratia marcescens* (Angerer *et al.*, 1992), *Yersinia pestis*, *Y.pseudotuberculosis* and *Y.enterocolitica* (Perry and Brubaker, 1979). Some of these heme-utilizing bacteria produce under low iron stress hemolysins that make heme more easily available to bacteria replicating *in vivo* (Waalwijk *et al.*, 1983). Recently, Daskaleros *et al.* (1991) isolated a cosmid clone from a *P.shigelloides* DNA library that allowed *Escherichia coli* cells to use hemin as a source of porphyrin and iron, but neither the gene(s) nor the mechanism of hemin

usage were addressed. Therefore, neither the mechanism of hemin uptake nor required components are known for any of these bacteria. However, Coulton and Pang (1983) showed that, at least in *H.influenzae* type *b*, heme does not merely diffuse into cells, but is taken up by an active process which in part satisfies the iron requirement of the cell.

Here, we present the first molecular characterization of hemin uptake in bacteria. The hemin uptake in *Y.enterocolitica* O:8 requires a hemin-specific outer membrane receptor. Hemin uptake is similar to other siderophore-iron scavenging uptake strategies in Gram-negative bacteria, namely it is a TonB-dependent translocation process across the outer membrane.

## Results

### *Isolation of a hemin uptake mutant of Y.enterocolitica O:8*

On iron limiting media like nutrient broth (NB) plates with 0.3 mM bipyridin, growth of *Y.enterocolitica* WA-C can be stimulated by hemin supplied on filter paper discs. To gain more insight into the nature of the hemin uptake system, we tested wether hemin uptake is a TonB-dependent process as has been observed for other iron transport systems and for vitamin $B_{12}$ uptake. The *tonB* mutant H2000 of *Y.enterocolitica* WA-C was isolated by selecting for simultaneous resistance to ferrimycin and albomycin. Ferrimycin and albomycin are iron-containing antibiotics that are internalized into the periplasmic space by the TonB-dependent process through the ferrioxamine outer membrane receptor (ferrimycin) and ferrichrome outer membrane receptor (albomycin). Simultaneous resistance to both antibiotics most probably would be the consequence of a TonB or ExbB/D mutation. The mutation in H2000 could be complemented by the *tonB* gene of *E.coli* (Koebnick *et al.*, 1992). This strain could not be fed with hemin as an iron source when grown on nutrient broth dipyridyl (NBD) plates, showing that hemin uptake in *Y.enterocolitica* is TonB dependent. The TonB dependence of hemin uptake also implied that there should be a specific receptor in the outer membrane for hemin that interacts with the TonB protein. To characterize the putative hemin receptor of *Y.enterocolitica* WA-C, we attempted to isolate mutants unable to grow on hemin as an iron source.

*Yersiniae* have two different ways of obtaining heme, by synthesizing it and by taking it up from the environment. We isolated a *hemA* mutant of *Y.enterocolitica* WA-C that was dependent on externally supplied 5-aminolevulinic acid, a biosynthetic precursor of heme. *Y.enterocolitica* was plated on minimal medium plates (with glucose as a carbon source) supplemented with 60 μg/ml of neomycin. A large proportion of the neomycin-resistant colonies were mutated in the heme biosynthesis pathway since such non-respiring cells do not efficiently transport neomycin into the cytoplasm (Beljanski and Beljanski, 1957; Sasarman *et al.*, 1968; Kanner and Gutnick, 1972). Several neomycin-resistant colonies were able to grow on tryptone−yeast extract (TY) plates only in the presence of 5-aminolevulinic acid, indicating that they were mutated in the early step of heme biosynthesis (Lewis *et al.*, 1991). The phenotype of these mutants corresponded to the phenotype of *hemA* mutants of *E.coli* with the difference that the putative *Y.enterocolitica* *hemA* mutants could grow in the presence of both hemin and 5-aminolevulinic acid. Both compounds rendered the *hemA* mutant (WA-C 11) as sensitive to neomycin as its parent.

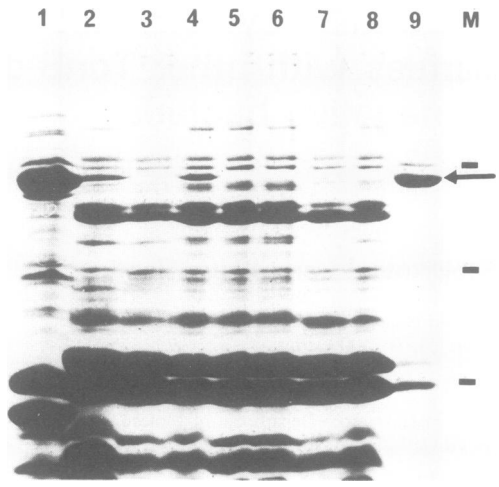


**Fig. 1.** SDS−PAGE of outer membranes from different *Y.enterocolitica* mutants. *E.coli* EB53 *hemA aroB* (pHEM76.53) (lane 1), *Y.enterocolitica* WA-C wild type (lane 2), WA-C 17 *hemA hemR* (lane 3), WA-C 1852 *fur* (lane 4), WA-C 1852I *fur hemR*::pHEM706 (lane 5), WA-C 1852H *fur hemR*::pHEM706 (lane 6), WA-C 103 *hemR*::pHEM706 (lane 7), WA-C 102 *hemR*::pHEM705.6 (lane 8) and WA-C 17 *hemA hemR* (pHEM101) (lane 9). Due to the strong overexpression of the HemR protein in WA-C 17 (pHEM101), only a small amount of the preparation was loaded on the gel. M, molecular weight markers: aldolase, 39.2 kDa; glutamate dehydrogenase, 55.5 kDa; fructose-6 phosphate kinase, 85.2 kDa.

In order to isolate a mutant in hemin uptake, we plated WA-C 11 on minimal medium supplemented with hemin and neomycin, and screened for neomycin resistance. Strain WA-C 17 was one of several neomycin-resistant small colonies that grew very poorly on TY plates supplemented with hemin (very small colonies appeared after several days of incubation), but was able to grow on plates supplemented with 5-aminolevulinic acid. This strain did not take up hemin as an iron source when grown on NBD plates supplemented with 5-aminolevulinic acid. However, it could grow on ferrichrome and ferrioxamine as iron sources and was pesticin-sensitive, which ruled out a mutation in the *tonB* or *exbBD* genes as a cause of the observed phenotype. Outer membranes of WA-C 17 *hemA hemR* mutant and parent strain WA-C, both grown under iron-limiting conditions (NB medium with 0.3 mM bipyridin), were isolated and the outer membrane protein patterns were compared (Figure 1). The only difference between these two strains was the absence of a 78 kDa protein from WA-C 17 (Figure 1, lane 3), which was strongly expressed in the WA-C strain (Figure 1, lane 2). The putative gene for the outer membrane hemin receptor was designated *hemR*.

### *Cloning of the hemin uptake system from Y.enterocolitica O:8*

To isolate the genes participating in hemin uptake, a pHC79 based cosmid library of *Y.enterocolitica* O:8 (Bäumler and Hantke, 1992) was transfected into strain AN344 of an *E.coli* K12 *hemA* mutant. This mutant could grow with 5-aminolevulinic acid, but could not use exogenously supplied hemin due to the impermeability of the outer membrane to hemin (McConville and Charles, 1979). *E.coli* harboring a cosmid

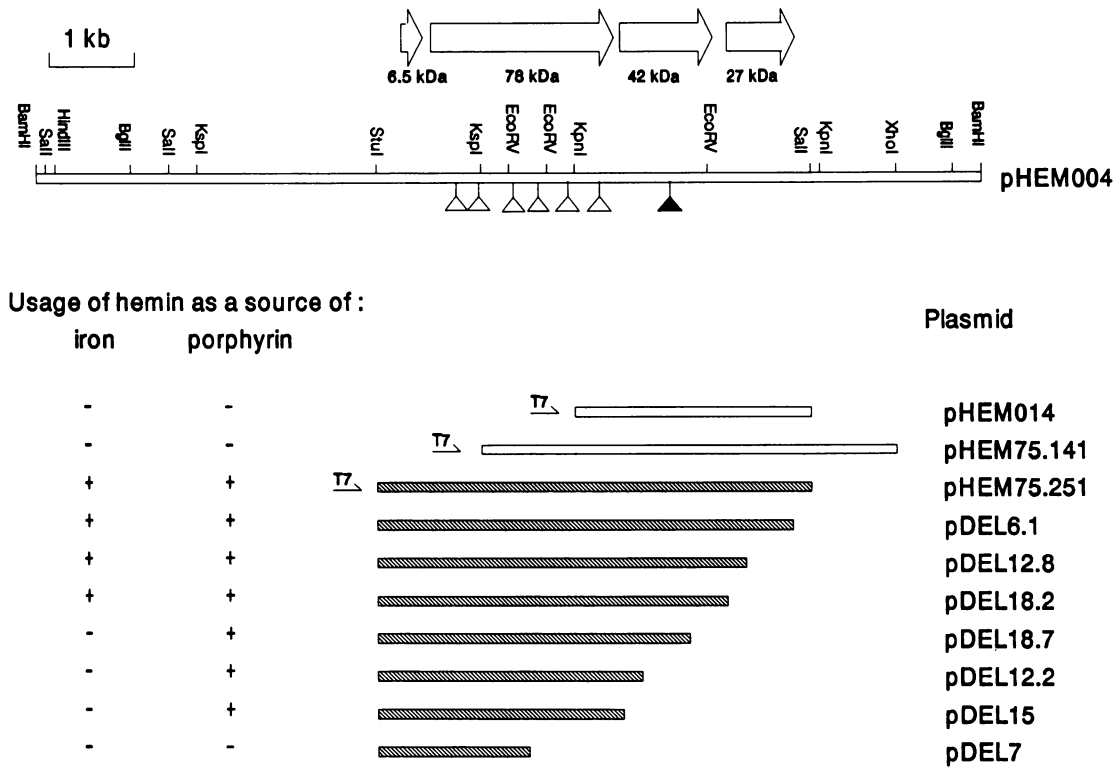


**Fig. 2.** Partial restriction map of plasmid pHEM004 and of different plasmid subclones carrying the hemin uptake operon. Open triangles indicate transposon insertions that inactivated the hemin uptake and the black triangle indicates the transposon insertion that inactivated only the usage of hemin as an iron source. Phenotypes of the plasmids were recorded after their transformation into *E.coli* EB53 *hemA aroB*. The 5.2 kb *Stu*I−*Sal*I fragment and 5.4 kb *Kpn*I−*Xho*I fragment of pHEM004 were first subcloned into plasmid pBC SK(+) and then as 5.2 kb *Bam*HI−*Sal*I and 5.4 kb *Sac*I−*Sal*I fragments, respectively, cloned into plasmid T7-5.

with the *hemR* gene should thus be able to use the exogenously supplied hemin. Indeed, we obtained colonies that grew on TY plates supplemented with hemin and ampicillin, but not on unsupplemented TY. Cosmids from four colonies were isolated and transformed into H1443 *aroB* and BR158 *aroB tonB* strains of *E.coli* K12. The *aroB* mutation rendered these strains unable to produce their own siderophore—enterochelin and therefore they were not able to grow under iron-limiting conditions unless supplied with some utilizable iron source. All four cosmids enabled the *aroB* strain to use hemin as an iron source, but neither of the *tonB* transformants could grow on NBD plates in the presence of hemin. DNA of one of the hemin-positive cosmids (pSI20) was digested with *Bam*HI, *Eco*RI, *Sal*I and *Hin*dIII and the fragments were cloned into the pBC SK(+) plasmid. One of the subclones (pHEM004) contained a 12.5 kb *Bam*HI fragment that enabled the *E.coli* EB53 *hemA aroB* strain to grow on TY and NBD plates supplemented with hemin, i.e. as porphyrin and iron sources, respectively. The restriction map of this subclone was constructed and the hemin locus mapped using transposon Tn*1732* (Ubben and Schmitt, 1986) (Figure 2). Strain RU664 carrying the thermosensitive plasmid pME305::Tn*1732* (Kan$^r$) was transformed with pHEM004 and then mated with EB53 *hemA aroB rpsL*. Chloramphenicol-, kanamycin- and streptomycin-resistant transconjugants were examined for hemin uptake. Of 69 transconjugants, six could not grow on TY plates supplemented with hemin and could not use hemin as an iron source. One transconjugant (pHEM004.9, Figure 2) used hemin as heme protein cofactor, but not as an iron source. Plasmid pHEM75.251, obtained by cloning

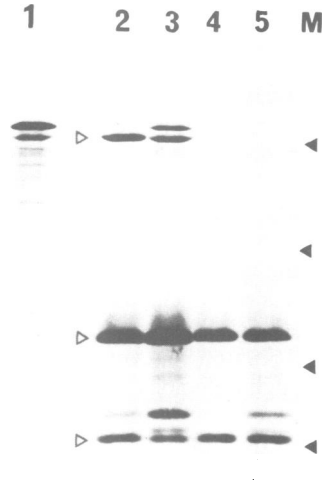


**Fig. 3.** SDS−PAGE of proteins encoded by the hemin uptake operon expressed from the phage T7 promoter. WM1576 (pHEM76.91) (lane 1), WM1576 (pHEM75.251) (lane 2), WM1576 (pHEM75.251) treated with sodium azide (lane 3), WM1576 (pHEM014) (lane 4) and WM1576 (pHEM014) treated with sodium azide (lane 5). Open triangles show the positions of HemR (78 kDa), HemS (42 kDa) and HemT (27 kDa). Closed triangles show the position of the molecular weight markers (pre-stained protein markers, Stratagene; 25.2, 33.5, 48.5 and 72.6 kDa).

the 5.2 kb *Stu*I−*Sal*I fragment from pHEM004 into the pT7-5 vector, enabled EB53 *hemA aroB* to use exogenous hemin when grown on TY and on NBD plates, i.e. as porphyrin and iron sources, respectively. On the other hand,

plasmid pHEM75.141, which carried the 5.4 kb *Kspl*−*Xho*I fragment of pHEM004 cloned into the same plasmid, and plasmid pHEM014, which carried the 3.1 kb large *Kpn*I−*Sal*I fragment of pHEM004, were unable to support growth of EB53 *hemA aroB* on hemin−NBD and hemin−TY plates. These data allowed us to localize the putative *hemR* gene to the first 3.1 kb of the 5.2 kb large *Stu*I−*Sal*I fragment of plasmid pHEM004. By using a new *Eco*RI restriction site introduced into the plasmid pHEM004 by the transposon Tn*1732*, we cloned the 3.1 kb large *Stu*I−*Eco*RI fragment containing the putative *hemR* gene of pHEM004.9:: Tn*1732* into plasmid pT7-6. The recombinant plasmid pHEM76.91 enabled EB53 *hemA aroB* to use exogenous hemin as a porphyrin source, but did not support growth on NBD−hemin. This result showed that utilization of hemin as an iron source required more genes than its use as a porphyrin source.

### Complementation of a hemR mutant

*Y.enterocolitica* O:8 *hemR* was complemented by the plasmid pHEM101, which carried the gene for a 78 kDa outer membrane protein (Figure 1, lane 9) and also with the plasmid pHEM75.53, which carried the complete hemin uptake operon (data not shown). *Y.enterocolitica* WA-C 17 *hemA hemR* (pHEM101) grew on TY plates supplemented with hemin (porphyrin source) and on hemin−NBD plates (iron source) with or without 5-aminolevulinic acid. The final proof that the gene on plasmid pHEM101 was indeed the *hemR* gene was obtained by insertional inactivation of the chromosomal *hemR* gene of *Y.enterocolitica*. The 0.9 kb *Kspl*−*Kpn*I fragment of pHEM101 was cloned into plasmid pGP704 (Miller and Mekalanos, 1988) creating the recombinant plasmid pHEM705. Plasmid pGP704 can replicate only in the presence of the Pir protein, whose gene is not present on the plasmid, but can be supplemented *in trans* by a second plasmid or a lambda *pir* lysogen (Kolter *et al.*, 1978). To improve selection for plasmid integration into the chromosome of WA-C, we introduced a spectinomycin cassette as a 1.9 kb *Hin*dIII fragment from pHP45Ω (Prentki and Krisch, 1984) into plasmid pHEM705, creating plasmid pHEM705.6. Plasmid pGP705.6 was introduced by conjugation into the WA-C strain of *Y.enterocolitica*. Since *Y.enterocolitica* does not contain the *pir* gene, spectinomycin- and ampicillin-resistant transconjugants could result only if the plasmid pHEM705.6 ($Sp^R$ $Amp^R$) was inserted into the chromosome through homology with *hemR*. Since plasmid pHEM705.6 contained truncated *hemR* at both ends, the resulting insertion of pHEM705.6 could inactivate chromosomal *hemR* (Miller and Mekalanos, 1988). The ampicillin- and spectinomycin-resistant transconjugant WA-C 102 was unable to use hemin as an iron source when grown on NBD plates and lacked the 78 kDa protein in the outer membrane (Figure 1, lane 8).

### Proteins involved in hemin uptake

The proteins encoded by the 5.2 kb *Stu*I−*Sal*I fragment and by the 3.1 kb *Kpn*I−*Sal*I fragment from pHEM004 were expressed *in vivo* from the plasmids pHEM75.251 and pHEM014, respectively, in which the genes were transcribed with the T7 RNA polymerase from the strong $\phi$10 T7 promoter gene (Tabor and Richardson, 1985). The 5.2 kb *Stu*I−*Sal*I fragment encoded at least three proteins of 78 (HemR), 42 (HemS) and 27 kDa (HemT) (Figure 3, lane 2). When sodium azide, a specific inhibitor of SecA (Fortin *et al.*, 1990), was added, the bands of the 78 and 27 kDa proteins were weaker and larger bands appeared (Figure 3, lane 3). The 3 kb *Kpn*I−*Sal*I fragment cloned into the pBC SK(+) in front of the T7 promoter encoded the 42 and 27 kDa proteins, of which the smaller one possessed a signal sequence, as determined by the sodium azide experiment (Figure 3, compare lane 4 with 5). Expression studies with the plasmids pHEM76.53, which carried the insert from plasmid pHEM75.251 in the opposite orientation to the T7 promoter, did not lead to any detectable proteins on SDS−PAGE gels (data not shown). Plasmid pHEM76.91, which carried a 3.1 kb *Stu*I−*Eco*RI fragment from pHEM004.9::Tn*1721*, encoded only a 78 kDa protein whose processing could be inhibited by sodium azide (Figure 3, lane 1). To determine which of the proteins are necessary for the assimilation of hemin as an iron source, deletions were introduced into the plasmid pHEM75.251 and the resulting deletion plasmids were expressed in the T7 promoter−polymerase (*in vivo*) system (Figure 4). Although the deletions in the plasmids pDEL6.1, pDEL12.8 and pDEL18.2 affected the *hemT* gene, which encoded the 27 kDa protein (Figure 4, lanes 3, 4, 5 and 6), strain EB53 *hemA aroB* transformed with these plasmids was still able to grow on NBD plates supplemented with hemin. On the other hand, a small deletion in the *hemS* gene encoding the 42 kDa protein resulted in the expression of a shortened protein (pDEL18.7, Figure 4, lane 7) and abolished the utilization of hemin as an iron source. However, plasmid pDEL18.7, when transformed into a *hemA aroB* background, enabled the strain to grow on TY plates supplemented with hemin. Plasmids pDEL15 and pDEL12.8, which have much larger deletions than plasmid pDEL18.7, conferred the same phenotype as plasmid pDEL18.7 when transformed into strain EB53 *hemA aroB* (Figure 4, lanes 8 and 9). Deletions affecting the *hemR* gene, which encodes the 78 kDa protein (plasmid pDEL7, lane 10), rendered these plasmids unable to support their *hemA aroB* hosts to use exogenously supplied hemin as a porphyrin source. These results clearly showed that cells need both the receptor HemR and the HemS protein for the usage of hemin-containing iron, but only the receptor HemR for the usage of hemin as a porphyrin source. Indeed, plasmid pHEM014, which encodes HemS and HemT proteins, transformed into strain EB53 *E.coli hemA aroB* (pHEM76.91) allowed it to use hemin as an iron source, although the strain alone was not able to grow on NBD−hemin plates. The same strain transformed with the plasmid pHEM100, which contained the 2 kb *Eco*RV fragment from pHEM004 encoding a truncated HemS protein (data not shown), did not allow growth on NBD−hemin plates. In both cases the transformants were plated on TY plates supplemented with hemin and chloramphenicol to ensure the presence of both plasmids in the cells.

### The expression of hemR gene is iron regulated

The HemR protein belongs to the iron regulated proteins of *Y.enterocolitica* O:8, since it is one of the outer membrane proteins whose expression is increased several-fold when cells are grown under iron limiting conditions. To study the regulation of the hemin uptake system, we constructed a transcriptional *hemR*−*phoA* fusion which was introduced into the chromosome of *Y.enterocolitica* WA-C and also into

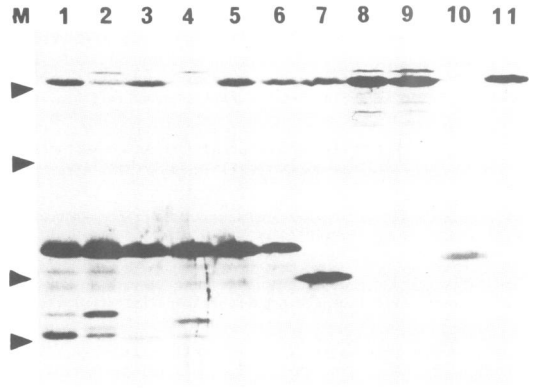


**Fig. 4.** SDS−PAGE of proteins encoded by different deletion derivatives of plasmid pHEM75.251 expressed from the T7 promoter *in vivo*. WM1576 (pHEM75.251) (lane 1), WM1576 (pHEM75.251) treated with sodium azide (lane 2), WM1576 (pDEL6.1) (lane 3), WM1576 (pDEL6.1) treated with sodium azide (lane 4), WM1576 (pDEL12.8) (lane 5), WM1576 (18.2) (lane 6), WM1576 (pDEL18.7) (lane 7), WM1576 (pDEL15) (lane 8), WM1576 (pDEL12.8) (lane 9) and WM1576 (pDEL7) (lane 10), WM1576 (pHEM76.91).
M, molecular weight markers. Pre-stained protein markers, Stratagene; 25.2, 33.5, 48.5 and 72.5 kDa.

a *Y.enterocolitica* WA-C *fur* mutant. To construct such a transcriptional fusion, we first introduced the 2.6 kb *Xho*I−*Sal*I DNA fragment of plasmid pUJ10, containing the promoterless *phoA* gene, into the *Sal*I linearized plasmid pBC SK(+). From one of the resulting recombinant plasmids the 2.6 kb *Kpn*I−*Xba*I DNA fragment containing *phoA* was ligated with the *Kpn*I and *Xba*I digested plasmid pHEM705.6, resulting in plasmid pHEM706. Plasmid pHEM706 contained the *phoA* gene directly 3′ to the 0.9 kb *Ksp*I−*Kpn*I DNA fragment of the *hemR* gene. This orientation would place the promoterless *phoA* gene under the control of the *hemR* promoter in the case of insertional inactivation of the chromosomal *hemR* with the plasmid pHEM706 (see above). After introduction of plasmid pHEM706 into the wild type and the *fur* mutant of *Y.enterocolitica* WA-C, several spectinomycin- and ampicillin-resistant colonies were checked for the hemin uptake phenotype. One hemin uptake-negative colony (WA-C 103) derived from WA-C and two independently hemin uptake-negative, *fur* mutant colonies (WA-C 1852I and WA-C 1852H) were used for further studies. Outer membrane preparations of these strains did not contain HemR (Figure 1, lanes 5, 6 and 7). Measurements of the alkaline phosphatase activity in cells grown in high (0.1 mM $FeCl_3$) and low iron medium (0.3 mM 2,2′-bipyridin), showed that the expression of the *phoA* gene is negatively regulated by the Fur repressor (Table I).

### *Nucleotide sequence of hemR gene*

The nucleotide sequence of the 3.04 kb *Stu*I−*Bal*I fragment containing the *hemR* gene and its promoter region was determined (Figure 5). The largest open reading frame preceded by a Shine−Dalgarno sequence (Shine and Dalgarno, 1974) begins at nucleotide 791 and ends with a stop codon at 2848. As predicted from the nucleotide sequence, HemR has a molecular weight of 75 060 Da and a typical signal sequence at its amino-terminal end. A well conserved Fur box is located 412 nucleotides from the beginning of the HemR coding region, starting at nucleotide

**Table I.** Alkaline phosphatase activity of *hemR::phoA* transcriptional fusions in wild type and *fur* mutants of *Y.enterocolitica*

| Strain | Alkaline phosphatase activity (U)[a] | |
|---|---|---|
| | $FeCl_3$ | 2,2′ bipyridin |
| WA-C *fur* 1852I | 123 | 252 |
| WA-C 103 | 16 | 195 |

[a]The cells were grown for 5 h in TY medium supplemented with 0.1 mM $FeCl_3$ or 0.3 mM bipyridin before the measurements were made. The experiments were repeated three times with essentially identical results. Values represented in the table are from one representative experiment. Values of the alkaline phosphatase activity of *Y.enterocolitica* wild type cells and those of *fur* mutants were measured to be between 9 and 10 U.

379 (Figure 5). An open reading frame from nucleotide 373 to 615 was found, which could encode a protein of 81 amino acids. However, no protein of this size was seen on SDS−PAGE gels when the entire operon was expressed on T7 plasmids (see Figures 2 and 3). After Tricine−SDS−PAGE (Schägger and von Jagow, 1987) a HemP protein of 6.5 kDa was expressed from the plasmids containing the *Stu*I−*Ksp*I (nucleotides 1−1440) fragment from pHEM101. This protein was not present when plasmids containing different deletions in the region (pKSP3.1, starting from nucleotide 498, and plasmid pKSP4.3, starting from nucleotide 870) were used in the T7 expression experiments (data not shown). At positions 2883−2911 of the nucleotide sequence, an inverted repeat (stem of 11 nucleotides and loop of seven nucleotides) was found which could act as a terminator of *hemR* transcription. At position 2956 the start of a new ORF, preceded by a typical Shine−Dalgarno sequence, was located which, according to the genetic experiments, may be the *hemS* gene (see above).

## Discussion

*E.coli* K12 and *Salmonella typhimurium* LT2 are normally unable to utilize hemin as a porphyrin source, since their outer membranes are impermeable to this substance. Only certain mutants with alterations in their outer membrane have been reported to be able to use hemin as a source for heme-containing proteins (Sasarman *et al.*, 1968; McConville and Charles, 1979). *Yersiniae* are able to use hemin not only as an iron source (see Introduction) but also as a porphyrin source, since the *hemA* mutant grew on exogenously supplied hemin. A *Y.enterocolitica* mutant unable to take up hemin was isolated with the neomycin procedure. A putative *hemR* mutant was complemented with the cloned *hemR* gene and a chromosomal *hemR* mutant was constructed with the aid of the cloned HemR receptor gene, proving the identity of the cloned and chromosomal *hemR* genes. TonB dependence of the hemin uptake in *Y.enterocolitica* and the characterization of the hemin receptor protein as a 78 kDa outer membrane iron-regulated protein clearly showed that this iron uptake system followed the pattern of all siderophore-iron assimilation systems described for Gram-negative bacteria up to now (Braun and Hantke, 1991).

A well conserved TonB box (Schramm *et al.*, 1987) was located in the amino-terminus of the HemR protein (positions 16−22 of the mature protein). It is identical to the TonB box of the *E.coli* Cir receptor (Nau and Konisky, 1989) and very similar to that of the *E.coli* BtuB receptor (Heller and Kadner, 1985) (Figure 6). Indeed, not only are the TonB

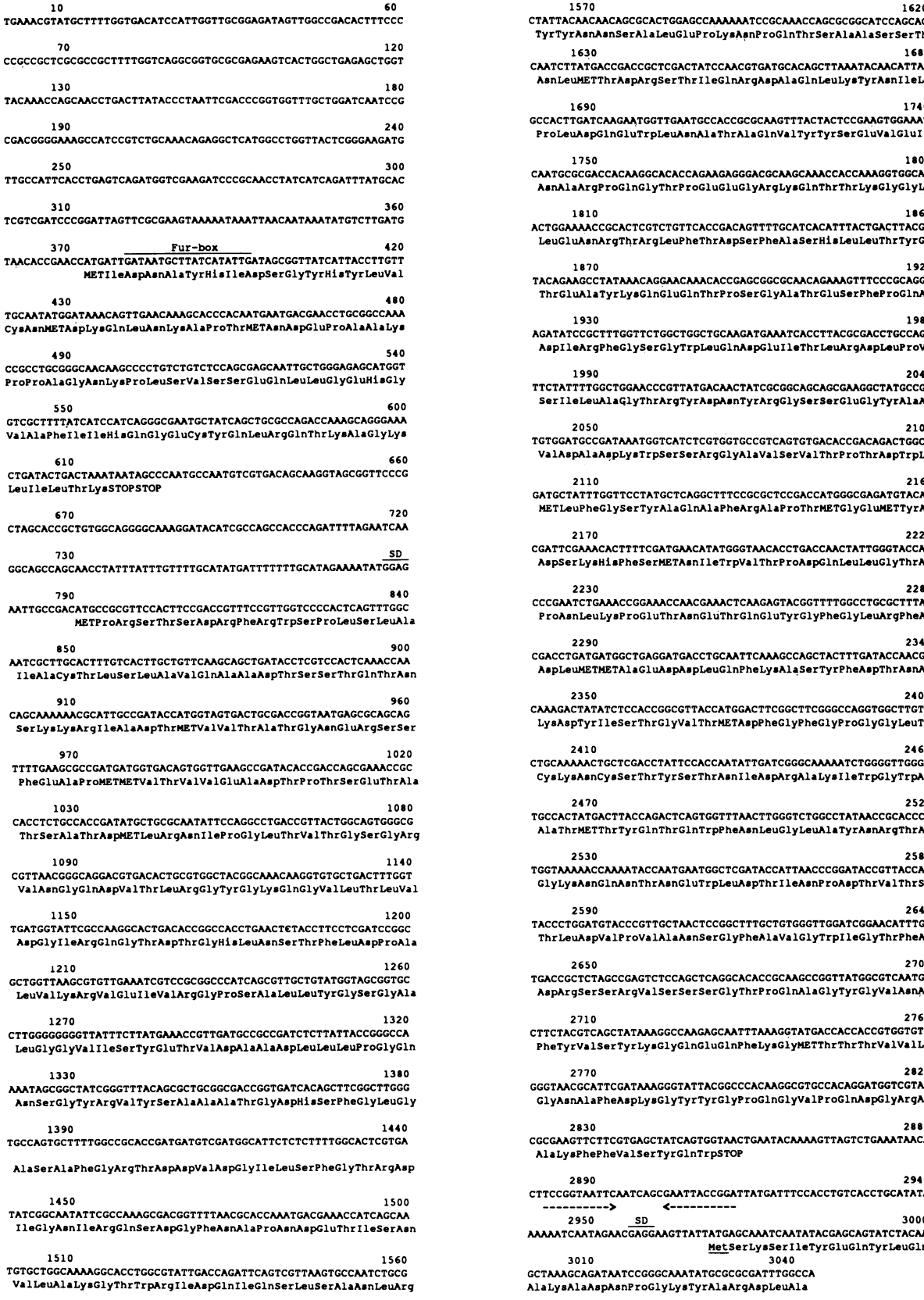


**Fig. 5.** Nucleotide sequence of the 3045 bp *StuI*−*BalI* fragment from pHEM101. The positions of the Shine−Dalgarno sequence (SD), the stop codon, a possible terminator (arrows) and a putative 'Fur box' are indicated.

boxes of these three proteins conserved, but also the entire amino acid sequences of these proteins are more closely related to each other than to other known receptors of Gram-negative bacteria (Figure 7). The HemR receptor shares the highest degree of homology with BtuB (23% amino acid identity), the vitamin $B_{12}$ receptor of *E.coli*. This similarity

```
        Signal sequence              1                TonB-box*
HemR  MPRSTSDRFRWSPLSLAIACTLSLAVQA ADTSSTQTNSKKRIADTMVVTA  22
BtuB  MIKKAS--LLTA--CSVTAFS-AW---A QDTS----------PDTLVVTA  12
Cir   MFRLNP--FVRVGLCLSAISC-AWPVLA VDDD----------GETMVVTA  12
                                   *::           :*:****
```

**Fig. 6.** Comparison of the amino-termini of HemR, BtuB and Cir proteins including the TonB box of the three proteins. A star indicates three identical residues, a colon indicates two identical residues in the three sequences. Amino acids from the mature proteins are written in bold letters.

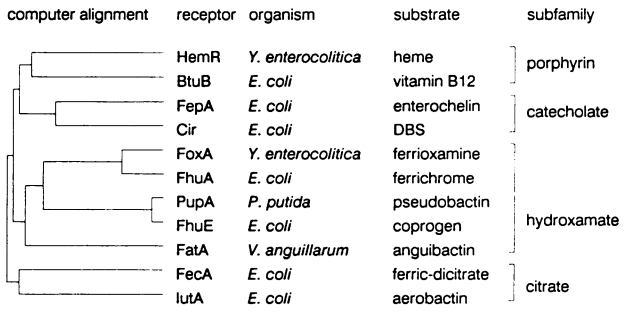


**Fig. 7.** Hypothetical phylogenetic tree derived from the amino acid sequence similarities of TonB-dependent receptors. The alignment was done using the program CLUSTAL from the program package PC/GENE (IntelliGenetics, Inc.). For legends concerning the sequences of the receptors see Bäumler and Hantke (1992).

may reflect a specificity for similar substrates, since both hemin and vitamin $B_{12}$ are porphyrin derivatives.

Highly pathogenic *Yersiniae* and certain *E.coli* strains are sensitive to the action of pesticin, the murein-degrading bacteriocin produced by *Y.pestis* (Brubaker, 1991). A putative pesticin receptor has not yet been identified, but some experimental data supported the hypothesis that the pesticin receptor is also the hemin receptor (Hu *et al.*, 1972). All HemR mutants isolated in this study were pesticin-sensitive, indicating that the pesticin and hemin receptors are two separate proteins.

The role of the hemin uptake system in the pathogenesis of yersiniosis is still an unresolved question. Jackson and Burrows (1956) showed that unpigmented strains of *Y.pestis* (which produce no pigmented colonies when grown on hemin-containing media at 26°C) were avirulent for mice unless the mice were overloaded with different iron-containing compounds. Later, Perry and Brubaker (1979) showed that although unpigmented and avirulent, these colonies were still able to use hemin as an iron source. *Y.enterocolitica* O:8 did not form pigmented colonies on hemin plates, nor did *E.coli* strains carrying the cloned hemin uptake determinant show any clear-cut pigmentation. Preliminary data on the virulence potential of a *Y. enterocolitica hemR* mutant (J.Heesemann, in preparation) show that, after intravenous inoculation, the HemR mutant is as virulent as the wild type strain. These results could be interpreted such that the siderophore uptake system of *Y.enterocolitica* O:8 (see Introduction) satisfies the need for iron after bacteria have entered the bloodstream (Heesemann, 1987). Thus, the mere possession of a hemin uptake system may not suffice to combat different hemin-scavenging mechanisms present in the body fluids (Eaton *et al.*, 1982; Otto *et al.*, 1992). Pathogenic factors such as the hemin storage capacity of *Y.pestis* (Perry *et al.*, 1990; Pendrak and Perry, 1991), utilization of haptoglobin bound haemoglobin in *Vibrio vulnificus* (Helms *et al.*, 1984) and the recently described hemin–hemopexin utilization system of *H.influenzae* type *b* (Hanson *et al.*, 1992), could all help bacteria to obtain hemin despite the host's hemin-scavenging strategies. The hemin uptake system could have greater importance for bacteria growing on mucosal surfaces where a large amount of hemin is present due to the desquamation of epithelial cells, and where hemin-scavenging systems of the host are not very efficient (Griffiths, 1987; Otto *et al.*, 1992).

The HemR protein belongs to the iron regulated proteins of *Y.enterocolitica*. A well conserved Fur binding site (Fur box) was located upstream of the *hemR* gene and a *hemR::phoA* transcriptional fusion was shown to be regulated by the *fur* gene product (Hantke, 1981). The nucleotide sequence of the promoter region revealed an open reading frame with three possible start codons. Judging by the molecular weight of the protein (6.5 kDa), the most probable start of translation is the second methionine at position 427 of the nucleotide sequence. Since the Fur box was found upstream of the *hemP* gene, its transcription should also be negatively regulated by Fur.

Plasmids that contained *hemP* and *HemR* genes conferred on transformants the ability to use hemin as a porphyrin source, which is in agreement with the fact that outer membrane represents a barrier to hemin uptake (see above). Since synthesis of cytochrome apoproteins is not coupled with hemin biosynthesis in *E.coli*, hemin can, once in the periplasm, be directly incorporated into cytochromes located in the cytoplasmic membrane (Haddock and Schairer, 1973). On the other hand, HemP, HemR and the product of the third gene, HemS, were shown to be necessary for the growth of plasmid-containing *hemA aroB* cells under iron-limiting conditions. The difference between porphyrin and iron utilization from hemin could be merely a quantitative one. The total iron content of *E.coli* was determined to be between 59 and 168 $\mu$g (per gram dry weight) of which hemin-iron represents only ~1% (Hartman and Braun, 1981; Matzanke *et al.*, 1989). Therefore, the amount of hemin-iron needed to satisfy the cell's need for iron is much larger than the amount of hemin (porphyrin) necessary to compensate for the cell's defect in heme biosynthesis. Most probably a heme-degrading activity is necessary for the usage of heme as an iron source. There is weak evidence for such an activity in *E.coli* K12. Eberspächer and Braun (1980) showed that heme compounds are degraded in an *E.coli hemA aroB* mutant grown in 5-aminolevulinic acid-free medium. However, the heme degrading activity has never been studied further. The HemS protein could be either a cytoplasmic membrane permease that transfers hemin into the cytoplasm or a hemin-degrading enzyme. The function of HemS is now under study.

The presence of the fourth protein, HemT, whose processing could be inhibited with sodium azide, was not necessary for either porphyrin or hemin-iron uptake in an *E.coli hemA aroB* strain. It is of interest to note that the $B_{12}$ uptake system of *E.coli*, whose receptor protein BtuB is HemR's closest 'relative' among sequenced receptors (Figure 7), also possesses a periplasmic binding protein (BtuE) that was not necessary for vitamin $B_{12}$ uptake (Rioux and Kadner, 1989). In conclusion, the hemin receptor of *Y.enterocolitica* O:8 was identified, and its gene cloned and sequenced. Hemin uptake in *Y.enterocolitica* was shown to be TonB-dependent, being similar to other siderophore and vitamin $B_{12}$ uptake systems.

**Table II.** Bacterial strains and plasmids

| Strain | Relevant genotype | Source |
|---|---|---|
| *E.coli* K12 | | |
| WM1576 | pGP1-2 | Tabor and Richardson (1985) |
| H1443 | *aroB* | Laboratory stock |
| BR158 | *tonB aroB* | Laboratory stock |
| EB53 | *aroB hemA* | Eberspächer and Braun (1980) |
| EB53-1 | EB53 $Str^R$ | This study |
| AN344 | *hemA* | Laboratory stock |
| SM10 | *recA* (*RP4-2-Tc::Mu*) (λ*pir*) | Miller and Mekalanos (1988) |
| JM101 | | Laboratory stock |
| Ru664 | pME305::Tn*1732* ($Kan^R$) | Ubben and Schmitt (1986) |
| *Y.enterocolitica* O:8 | | |
| WA-C | plasmidless derivative | Heesemann (1987) |
| H2000 | WA-C *tonB* | K.Hantke |
| WA-C 1852 | *fur-5* | K.Hantke |
| WA-C 11 | WA-C *hemA* | This study |
| WA-C 17 | WA-C 11 *hemR* | This study |
| WA-C 102 | *hemR*::pHEM705.6 | This study |
| WA-C 103 | *hemR*::pHEM706 | This study |
| WA-C 1852I | *fur hemR*::pHEM706 | This study |
| WA-C 1852H | *fur hemR*::pHEM706 | This study |
| Plasmids | | |
| pBC SK(+) | $Chl^R$ | Laboratory stock |
| pT7-5 | $Amp^R$ | Tabor and Richardson (1985) |
| pT7-6 | $Amp^R$ | Tabor and Richardson (1985) |
| pGP704 | *oriR6K* $Amp^R$ | Miller and Mekalanos (1988) |
| pSI20 | pHC79 $Amp^R$, *hemP hemR hemS hemT* | This study |
| pHEM004 | 12.5 kb *Bam*HI fragment of pSI20 cloned in pBC | This study |
| pHEM004.9 | pHEM004 *hemS*::Tn*1732* | This study |
| pHEM75.251 | 5.2 kb *Stu*I−*Sal*I fragment of pHEM004 cloned in pT7-5 | This study |
| pHEM76.53 | 5.2 kb *Stu*I−*Sal*I fragment cloned in pT7-6 | This study |
| pHEM75.141 | 5.4 kb *Ksp*I−*Xho*I fragment of pHEM004 cloned in pT7-5 | This study |
| pHEM014 | 3.1 kb *Kpn*I−*Sal*I fragment of pHEM004 cloned in pBC | This study |
| pHEM76.91 | 3.1 kb *Eco*RI−*Stu*I fragment of pHEM004.9 cloned in pT7-6 | This study |
| pHEM100 | 2 kb *Eco*RV fragment of pHEM75.251 cloned in pBC | This study |
| pHEM101 | 3.1 kb *Eco*RI−*Bam*HI fragment of pHEM76.91 cloned in pBC | This study |
| pHEM705 | 0.9 kb *Ksp*I−*Kpn*I fragment of pHEM76.53 cloned in pGP704 | This study |
| pHP45Ω | $Sp^RSm^R$ | Prentki and Krisch (1984) |
| pHEM705.6 | 1.9 b *Hin*dIII fragment of pHP45Ω cloned in pHEM705 | This study |
| pUJ10 | *phoA lacZ* $Amp^R$ | De Lorenzo *et al.* (1990) |
| pHEM706 | 2.6 kb *Kpn*I−*Xba*I fragment of pUJ10 cloned in pHEM705.6 | This study |

## Materials and methods

### Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table II.

### Media and growth conditions

*E.coli* strains were routinely cultured aerobically at 37°C in LB or TY medium (Sambrook *et al.*, 1989) except for strains containing plasmid pGP1-2, which were grown at 28°C. *Y.enterocolitica* strains were grown aerobically at 28°C in TY medium or on TY plates. *HemA* and *hemR* mutants of *Y.enterocolitica* were isolated on glucose M9 minimal plates supplemented with 60 mg/l of neomycin. Antibiotics were used at the following concentrations: ampicillin, 50−100 mg/l; neomycin, 75 mg/l; spectinomycin, 70 mg/l; chloramphenicol, 30 mg/l; streptomycin, 100 mg/l.5-amino-levulinic acid (Sigma) was used at a final concentration of 30 mg/l and bovine hemin chloride (Sigma) was dissolved in 20 mM NaOH to a final concentration of 30 mg/l. Iron-restricted growth conditions were achieved by growing the cells in NBD medium or on NBD plates (8 g nutrient broth, 5 g NaCl and 15 g of agar per litre) supplemented with 0.3 mM 2,2′ bipyridin. Growth stimulation of iron-restricted *Y.enterocolitica* or *E.coli* cells by hemin was tested by applying the strain in 3 ml of water soft agar on NBD plates. Filter paper discs impregnated with 20 μl of 3 mg/ml hemin stock solution were placed on the NBD plate with the strain and growth stimulation was recorded after overnight incubation at 37°C.

### Alkaline phosphatase assay

The enzymatic activity of alkaline phosphatase operon fusions in *Y.enterocolitica* was determined as described by Brickman and Beckwith (1975). Overnight cultures of the strain were diluted 1:100 in TY medium and aerated for 2 h at 37°C. The iron limiting conditions were created by addition of 0.3 mM 2,2′ bipyridin and iron sufficient growth conditions by addition of 0.1 mM $FeCl_3$ to the culture. The cultures were incubated for another 5 h at 37°C and then the enzymatic activity of alkaline phosphatase was determined from the measurement of hydrolysis of 4-nitro-phenyl phosphate (dinatrium salt, Merck) by permeabilized cells (Miller, 1972).

### Recombinant DNA techniques

Standard methods for plasmid DNA isolation, restriction endonuclease analyses and ligation were carried out according to Sambrook *et al.* (1989). The *Y.enterocolitica* cosmid library described previously by Bäumler and Hantke (1992) was used. Electroporation of *Y.enterocolitica* was done essentially according to the manufacturer's instructions (Bio-Rad).

### DNA sequence determination

The DNA sequence of the *hemR* gene was determined by the dideoxy chain termination method using Sequenase$^R$ version 2.0 kit (USB) and an LKB electrophoresis system. Plasmid subclones necessary for sequencing were created by nested deletions of plasmid pHEM101 using Erase-a-Base$^R$ System (Promega) and by using different restriction sites within the *hemR* gene. Both strands were sequenced.

***Expression of plasmid encoded proteins***

DNA fragments were cloned into the pT7-5, pT7-6 and pBC upstream of the T7 RNA polymerase promoter (Tabor and Richardson, 1985). Recombinant plasmids were then transformed into *E.coli* WM1576, which contained the plasmid encoded (pGP1-2) T7 RNA polymerase gene under the lambda $P_L$ promoter control. Transcription of the T7 RNA polymerase from the plasmid pGP1-2 is repressed by the thermosensitive lambda cI857 repressor which is also present on pGP1-2. Plasmid-encoded proteins were labelled with [$^{35}$S]methionine after inhibition of *E.coli* RNA polymerase with rifampicin (200 mg/l, Sigma) and expression of T7 RNA polymerase by temperature induction. Whole cell proteins were separated by SDS–PAGE and bands were identified by fluorography. When necessary, the inhibition of the *E.coli* secretion apparatus was performed by adding sodium azide (5 mM final concentration) before radioactive labelling in order to enrich for the polypeptides with signal sequences (Fortin *et al.*, 1990). Outer membranes of unlabelled cells were isolated by the Triton X-100–$MgCl_2$ procedure as described by Hantke (1981).

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## Noted added in proof

The sequence data of *hemP* and *hemR* have been deposited in the EMBL Data Library under the accession number X68147.