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13. ABSTRACT (Maximum 200 words)

In this investigation, we have demonstrated that DNA from A. magnetotacticum can be functionally expressed in E. coli. However, for the same amino acids, the codons used by this bacterium are often different from those used by E. coli.

The mechanism of iron-uptake in A. magnetotacticum is not mediated by a siderophore compound. However, the common pathway leading to the synthesis of aromatic amino acids and enterochelin, the E. coli siderophore, is conserved.

We have isolated a mutant strain that is no longer magnetotactic. A major cytoplasmic protein present in the magnetotactic strain is absent in the mutant strain. We have purified and partially characterized this protein.

We have cloned a fragment from DNA of A. magnetotacticum that allows growth in the presence of 2,2'-dipyridyl. Cells remove the chelator from the medium and gradually turn pink in color (dipyridyl + Fe^{2+} is pink).

We have been able to grow A. magnetotacticum on solid medium under aerobic conditions by adding Oxyrase™ to the medium.

14. SUBJECT TERMS
Aquaspirillum magnetotacticum, magnetic bacteria, iron-uptake, siderophore

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FINAL REPORT: CONTRACT N00014-89-C-0085

CONTRACT TITLE: GENETIC ENGINEERING OF SINGLE-DOMAIN MAGNETIC PARTICLES

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BACKGROUND AND OBJECTIVES

Magnetotactic bacteria selectively synthesize nanometer-sized, single-domain magnetic particles as part of their normal life cycle. These bacteria orient themselves along the magnetic field lines of earth and direct their motion toward habitats most suitable for their survival. Most of our knowledge of magnetotaxis in bacteria comes from studying a bacterium named *Aquaspirillum magnetotacticum.* This bipolarly flagellated, freshwater magnetotactic spirillum is microaerophilic and is grown in pure culture.

Previous studies, in particular the work of Paoletti *et al.* (J. Bacteriol., 167: 73, 1986), had established that iron-uptake in *A. magnetotacticum* is mediated by a siderophore of hydroxamate type. The original objective of this proposal was to clone and characterize the genes of this iron-uptake system by using two different approaches: (i) screening the genomic libraries prepared from the DNA of this bacterium, and (ii) identifying and cloning the DNA sequences of *A. magnetotacticum* that are homologous to genes associated with high-affinity iron-uptake system of other microorganisms. In the course of our studies, we have conducted exhaustive search for this iron-uptake system by screening the genomic libraries of *A. magnetotacticum* and have concluded that no siderophore compound is produced by *A. magnetotacticum.* Our results are supported by the finding that no siderophore has yet been found in other anaerobic microorganisms.

Although *A. magnetotacticum* does not synthesize a siderophore compound, it possesses the common biosynthetic pathway leading to the synthesis of aromatic amino acids and enterochelin, the native siderophore of *E. coli.* We have demonstrated that by cloning and characterizing *aroD*, one of the genes of this biosynthetic pathway. This, together with our other results are summarized below.

APPROACH

Recombinant DNA techniques were used to conduct genetic studies in this bacterium. Genomic libraries were constructed and screened for either (i) a siderophore-mediated iron-uptake system or (ii) sequences homologous to genes associated with high-affinity iron-uptake system of other microorganisms. In our initial studies, to demonstrate the feasibility of using recombinant DNA techniques, the libraries were screened for sequences that would support growth of auxotrophic strains of *E. coli* in the absence of the required nutrients. The use of Oxyrase^{TM} in culture medium allowed us to grow *A. magnetotacticum* on solid medium under aerobic incubation conditions.

RESULTS

Gene Complementation

In our early experiments, to demonstrate the feasibility of using genetic engineering techniques in the study of iron assimilation in *A magnetotacticum*, we have shown that auxotrophic strains of *E. coli* can be complemented with DNA fragments isolated from *A. magnetotacticum*. In this study, gene libraries constructed from the DNA of *A. magnetotacticum* were screened for sequences that would allow growth of auxotrophic strains of *E. coli* in the absence of the required nutrients. We were successful in isolating fragments that complemented the *leuB6* (a point mutation) and *proA2* (a deletion mutation) mutations of strain HB101 and the *thr-1* (a point mutation) mutation of strain AB1133. A manuscript describing this work is attached

Codon Usage in *A. magnetotacticum*

In order to obtain information about the codons used by *A. magnetotacticum*, we have cloned and sequenced a gene unrelated to iron-uptake genes. The knowledge of codon usage proved to be valuable to us for the construction of specific probes needed in various screening experiments. We chose the gene analogous to the *recA* sequence of *E. coli*. Because of its importance to the cell, we assumed that this gene must have been preserved evolutionarily among all bacterial species. The *recA* gene of *E. coli* is involved in homologous recombination and DNA repair. It also regulates the expression of a number of genes scattered in the chromosome. We were successful in isolating and characterizing a *recA*-like sequence from the genomic library of *A magnetotacticum*. This gene is highly homologous to the *recA* sequence of *E. coli*.

The entire *recA* sequence of *A. magnetotacticum* has been sequenced and analyzed for the frequency of codons used. The codon preference of *A. magnetotacticum* for certain amino acids was found to be markedly different from that of *E. coli*. The information obtained from this study has been used for the construction of one of the probes used for cloning the coding sequence of the iron-binding protein described below. Two manuscripts describing this work are included.

Screening the Genomic Library for a Siderophore-Mediated Iron-Uptake System

We have screened genomic libraries prepared from the DNA of *A. magnetotacticum* for the genes of a siderophore-mediated iron-uptake system. A hydroxamate-mediated iron-uptake system had previously been reported to exist in this bacterium (Paoletti and Blakemore, J. Bacteriol. 167: 73, 1986). The recombinant cosmids were propagated in an iron-uptake-deficient strain of *E. coli* host strain. The library clones were plated on a medium containing a dye-iron complex (Schwyn and Neilands, Anal. Biochem. 160: 47, 1987) that turns from blue to orange in the presence of a chelating molecule. In spite of our extensive screening, we were unable to identify a siderophore-producing molecule. Out of 10,000 colonies tested, none changed the color of the medium.

One possibility is that the iron-uptake genes are scattered in the chromosome of *A. magnetotacticum.* In that case, one should be able to detect the siderophore or its binding activity in the supernatant culture fluids of the organism. We used the Csaky test (Gillman et. al., Anal. Chem. 53:, 841, 1981) for the detection of the hydroxamate-type siderophore in the supernatant culture fluids. In this test also, we did not observe any iron-binding activity, even when the supernatant was concentrated by about 20-fold.

Isolation of 2,2'-dipyridyl-resistant colonies that become"pink" when grown in the presence of the chelator.

Since no positive results were obtained in screening the libraries on the Schwyn and Neilands medium (described above), we selected 2,2'-dipyridyl as the chelating agent in the screening experiments.. Two clones were isolated from a cosmid library of *A. magnetotacticum* that grew in the presence of inhibitory concentrations (250 uM) of 2,2'-dipyridyl. These colonies turn "pink" when grown in a 2,2'-dipyridyl-containing medium. Upon streaking on L+2,2'-dipyridyl plates (this medium has a salmon color before inoculation), the pink color of the cells intensifies as the color of the plate changes from salmon pink to yellow (the color of L-agar medium). The library clones are nonmagnetotactic and appear normal (not pink) when grown in the absence of 2,2'-dipyridyl. We have verified the identity of the "pink" cells as library clones by showing that these cells i) have the same nutritional requirements and colony morphology as the host strain, ii) carry the cosimd sequence, and iii) carry sequences from the genomic material of (DNA) of *A. magnetotacticum* . These clones do not utilize 2,2'-Dipyridyl as sole carbon source and do not synthesize any siderophore as tested by the universal assay medium of Schwyn and Neilands.

We have also demonstrated that the observed phenotypes are associated with the cloned sequence. This has been accomplished by packaging the recombinant cosmid purified from one of the clones and transducing it into the original host, *E. coli* HB101 *fepA entA*. The host strain is iron-uptake deficient because it lacks the receptor (*fepA*) and one of the biosynthetic gene (*entA*) of enterochelin. Transductants grew in the presence of inhibitory concentrations of the chelator and formed "pink" colonies.

Plasmid analysis has indicated that the two library clones carry identical recombinant cosmids (as indicated by restriction analysis) with an insert of about 30 kb in size. Analysis of total cell extract indicates that at least two distinct protein bands with molecular weights of about 45, and 26 kDa, are produced by the two recombinant clones. The expression of the 26 KDa protein increases when cells are cultured in the presence of 2,2'-dipyridyl. The other gene products (if any other than the 45 and 26 KDa) are masked by the cell extract proteins.

Screening for Sequences Homologous to the Iron-Uptake Related Genes of *E. coli*

We have conducted Southern blot experiments with the digested DNA of *A. magnetotacticum* using a number of iron-uptake associated genes of *E. coli* as probe. The sequences that we have examined so far include, the entire enterochelin and aerobactin operons of *E. coli*, the receptor gene of ferrichrome-mediated iron-uptake gene (*fhuA*), a ferrichrome-mediated iron-uptake gene (*fhuB*), the consensus FUR binding site, the *tonB* gene, and the *btuB* gene.

In these experiments, we were able to identify fragments that hybridized with the *tonB*-specific probes. However, upon cloning and sequencing, no significant homology was detected between the cloned fragments and the *tonB* sequence of *E. coli*. We obtained similar results using the polymerase chain reactior (PCR) for cloning the *tonB*-like gene of *A. magnetotacticum*. The PCR primers were constructed complementary to the 5' and 3' regions of the *tonB* gene of *E. coli*. We obtained three major products, one a fragment with approximately the same size as the PCR product of the *tonB* gene of *E. coli*. Upon subcloning and sequencing, no significant homology was obtained between the PCR-amplified fragment of *A. magnetotacticum* and the *tonB* gene of *E. coli*.

Cloning of a Sequence of *A. magnetotacticum* that complements the *aroD* gene of *E. coli*

Since no homology was detected between the iron uptake genes of *E. coli* and the sequences of *A. magnetotacticum* (described above), we decided to screen for the presence of *aroD* gene sequence. The *aroD* gene of *E. coli* codes for the enzyme 3-dehydroquinase which catalyzes the third step of the early common pathway for the biosynthesis of chorismic acid, precursor of aromatic amino acids and enterochelin. We screened the library of *A. magnetotacticum* for sequences that would allow growth of *E. coli*, CL451 (=LE392 *aroD*::Tn10) in absence of aromatic amino acids. We were successful in cloning a sequence that conferred Aro^+ phenotype upon transfer into the *aro*$^-$ mutant strain. The cloned sequence, which was about 2 kb in size, also complemented the aromatic-metabolite-requirements of the *aroD* mutant strains of *Salmonella typhimurium*. A manuscript describing this work is attached.

Isolation of a nonmagnetotactic mutant strain of *A. magnetotacticum*

We have isolated a mutant strain of *A. magnetotacticum* that no longer is magnetotactic. This mutant strain, isolated during the process of an electroporation study, does not carry any plasmid and is sensitive to

kanamycin (the resistance marker of the electroporating plasmid). The nonmagnetic mutant strain has been stable and has not reverted to wild type phenotype upon subculturing. Electronmicroscopic studies have revealed that the nonmagnetic cells do not produce any magnetic particles. The protein profile of the mutant differes from that of the parent strain. Several high-molecular weight proteins (about 60-70 kDa) are produced by the mutant but not by the parent strain. The most striking difference is a major cytoplasmic protein (of about 16.5 KDa) present in the magnetic cells that is absent in the nonmagnetic mutant strain.

Purification of an iron-binding protein from the cytoplasmic fraction of *A. magnetotacticum.*

Using immobilized metal-ion affinity chromatography, we have been able to identify a number of proteins from the total cell extract of *A. magnetotacticum* that show strong binding affinity for metal iron. One of these proteins, associated with the cytoplasmic fraction, has been purified to homogeneity by reverse phase high-performance liquid chromatography (HPLC). This protein which accounts for about 16% of the cytoplasmic protein fraction is absent in the nonmagnetic mutant strain described above. It has a molecular weight of about 16.5 kDa and pI of 6.2. The amino acid composition of this protein (shown below) was determined and compared with total protein of *E. coli* :

Residue	%Amino Acids	
	A. magnetotacticum	*E. coli*
Asx	12.57	10.68
Thr	7.51	5.74
Ser	9.51	4.83
Glx	10.54	11.91
Pro	3.79	4.21
Gly	3.39	10.17
Ala	10.40	11.16
Val	7.71	7.62
Ile	2.53	5.45
Leu	2.44	8.87
Tyr	4.93	2.74
Phe	2.66	3.47
His	4.47	2.00
Lys	8.61	6.61
Arg	3.58	5.55
MetSO2	3.15	2.27
Cysteic	2.20	0.95

The N-terminal amino acid sequence of this protein and two internal peptides were obtained. Two oligonucleotide fragments complementary to the N-terminal region and two oligomers complementary to the internal sequences were constructed and used as probes in screening a genomic library. One of the probes complementary to the N-terminal region was a degenerate oligomer and the other was designed on the basis of best codon usage of *A. magnetotacticum*. Hybridization experiments revealed a band of about 3 kb that hybridized strongly with the two internal probes. This band was subcloned into bacteriophage M13 DNA and used for sequencing. So far, we have sequenced 1114 bases of this fragment.

Growth of A. magnetotacticum in the presence of Oxyrase™

A. magnetotacticum is a microaerophillic organism requiring 0.5-0.6% oxygen for growth and synthesis of magnetic particles. In our laboratory, this bacterium is routinely grown in nitrogen-gased liquic medium prepared in a sealed bottle or in solid medium in a chamber with about 0.6% oxygen. Because these procedures are laborious and not suitable for large-scale experiments, it was desirable to identify simpler methods for growing this organism.

Oxyrase (trade name) is an *E. coli* enzyme which in the presence of lactate or succinate removes oxygen from the medium. (The identity of the enzyme is not disclosed by the supplier, Oxyrase, Inc., Ashland, OH). We investigated the effect of various concentrations of Oxyrase on growth of *A. magnetotacticum* on solid medium. Minimal agar medium containing succinate were autoclaved and cooled to 42°C. The medium was then inoculated with the bacterial cells and poured in glass petri plates after the addition of the appropriate concentration of Oxyrase. Plates were incubated at 30°C under aerobic environment. Our results indicated that Oxyrase at concentrations between 0.06 to 0.12 units/ml promoted growth of *A. magnetotacticum*. Higher concentrations of Oxyrase had inhibitory effects. Under the microscope, *A. magnetotacticum* colonies had "snow-flake" appearance with a dark brown center.

PUBLICATIONS

1. Waleh, N.S. 1988. Functional expression of *Aquaspirillum magnetotacticum* genes in *Escherichia coli* K12. Mol. Gen. Genet. **214**:592-594.

2. Berson, A.E., D.V. Hudson, and N.S. Waleh. 1989. Cloning and characterization of the *recA* gene of *Aquaspirillum magnetotacticum.* Arch. Microbiol. **152**:567-571.

3. Berson, A.E., R.M. Peters, and N.S. Waleh. 1990. Nucleotide sequence of the *recA* gene of *Aquaspirillum magnetotacticum.* Nucl. Acids Res. **18**:675.

4. Berson, A.E. and N.S. Waleh. 1990. Molecular cloning of a sequence of *Aquaspirillum magnetotacticumm* that allows growth in the presence of 2,2'-dipyridyl. Annu. Mtg. Am. Soc. Microbiol.

5. Berson, A.E., D.V. Hudson, and N.S. Waleh. 1991. Cloning of a sequence of *Aquaspirillum magnetotacticum* that complements the *aroD* gene of *Escherichia coli.* Mol. Microbiol. **5**:2261-2264.

6. Waleh, N.S., D.V. Hudson, and A.E. Berson. 1991. Isolation of an iron-binding protein from the cytoplasmic fraction of *Aquaspirillum magnetotacticum.* Annu. Mtg. Am. Soc. Microbiol.

PERSONNEL SUPPORTED

Peters, R.M., an undergraduate student, male caucasion, supported for 2.5 months in 1990.

Mol Gen Genet (1988) 214:592–594

Functional expression of *Aquaspirillum magnetotacticum* genes in *Escherichia coli* K12

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Summary. Gene libraries from the magnetotactic bacterium, *Aquaspirillum magnetotacticum* were constructed in *Escherichia coli* with cosmids pLAFR3 and c2RB as vectors. Recombinant cosmids able to complement the *thr-1*, *leuB*, and *proA* mutations of the host were identified. The Pro$^+$ recombinant cosmid restored wild-type phenotype in *proA* and *proB* but not in the *proC* mutants of *E. coli*. The results of restriction endonuclease digestion and Southern hybridization analysis indicate that the relevent *leu* and *pro* biosynthetic genes of *A. magnetotacticum* are not closely linked on the chromosome.

Key words: Gene library – Recombinant cosmid – *Aquaspirillum magnetotacticum* – magnetotaxis

Bacteria that use the earth's magnetic field to direct their motion toward habitats most suitable for their survival were described over a decade ago (Blakemore 1975). These bacteria were shown to be globally distributed and have now been isolated from various marine and freshwater aquatic environments in both the northern (Blakemore 1975; Moench and Konetzka 1978; Frankel et al. 1979) and southern hemispheres (Blakemore et al. 1980) as well as near the equator (Frankel et al. 1981).

Most of our knowledge of magnetotaxis in bacteria comes from studying the bacterium named *Aquaspirillum magnetotacticum* strain MS-1 (Maratea and Blakemore 1981). This bipolarly flagellated, freshwater magnetotactic spirillum is so far the only species that has been grown in pure culture in a chemically defined medium (Blakemore et al. 1979). In the presence of ferric quinate, an iron source, this bacterium produces intracellular single-domain magnetic particles and becomes responsive to a magnetic field.

Very little is known about the genetic structure of *A. magnetotacticum*. Genetic studies of this bacterium are complicated because *A. magnetotacticum* is fastidious and requires elaborate techniques for growth and maintenance. Moreover, none of the genetic transfer systems (conjugation, transduction, or transformation) used in other bacteria are, at present, available for this organism. Recombinant DNA techniques provide an attractive alternative for initiation of genetic studies of this magnetotactic bacterium. In experiments to test the feasibility of using such techniques, it was decided to see whether auxotrophic strains of *Escherichia coli* K12 could be complemented with DNA fragments from *A. magnetotacticum*.

For this purpose, a gene library was constructed in a broad host-range cosmid, pLAFR3 (Staskawicz et al. 1987). This cloning vector is 22 kb long, has one *cos* site, and confers tetracycline resistance (Tc^r). *A. magnetotacticum* was grown as described by Blakemore et al. (1979) and its chromosomal DNA was isolated by the procedure of Rodriguez and Tait (1983). The DNA was partially digested with *Sau*3A endonuclease and ligated with *Bam*HI-digested cosmid pLAFR3 DNA, which had been treated with calf intestinal phosphatase, extracted with chloroform and isoamyl alcohol, and precipitated with ethanol. The total DNA concentration in the ligation reaction with T4 DNA ligase was about 45 μg/ml, and the ratio of insert to vector ends was 3:1. The ligation reactions were carried out at 16° C, overnight. The ligated DNA was packaged in bacteriophage lambda heads (using the commercially available packaging extract Gold Giga Pack, Stratagene Cloning Systems, San Diego, Calif) and used to transduce *E. coli* strain AB1133 (Table 1). *E. coli* AB1133 is wild-type for the restriction system of *E. coli* K12, and restricted the DNA of *A. magnetotacticum*. Each microgram of DNA yielded less than 100 Tc^r transductants that carried a recombinant cosmid. The *Eco*RI digestion patterns of the plasmids from 20 Tc^r clones taken at random indicated an isert size for 8 plasmids ranging from 5–25 kb with 12 clones carrying only the vector without insert. The Tc^r clones were screened for Thr$^+$ and Pro$^+$ phenotypes. In AB1133, *proA2* is a deletion and *thr-1* is a point mutation at an unidentified locus in the threonine biosynthetic operon (Taylor and Thoman 1964). Among 629 independently isolated Tc^r clones, 2 were found that grew in the absence of threonine. None of the clones, however, were Pro$^+$. The Thr$^+$ clones lost their ability to

Table 1. Bacterial strains

Strain	Genotype	Reference
AB1133	F$^-$ *thr-1 leuB6 proA2 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 λ$^-$ SupE44*	Taylor and Thoman (1964)
HB101	F$^-$ *hsd20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 xyl1 mtl1 supE44 str λ$^-$*	Bolivar et al. (1977)
AD37	*Δ(gpt-lac)5 thi-1 mal-24 spcA12 λ$^-$*	Datta et al. (1987)
X340	*proB28 metB1 relA1 spcT1 λ$^-$*	Datta et al. (1987)
X342	*proC29 metB1 relA1 spcT1 λ$^-$*	Datta et al. (1987)

Table 2. Results of selection for Thr$^+$ in AB1133

Cosmid	Number of transformants per plate[a]	
	L[b] + Tc	Minimal[c] + Tc
pLAFR3	> 1000	0, 1, 0, 0
Thr$^+$-1	356, 351, 400, 405	400, 410, 395, 360
Thr$^+$-2	320, 375, 295, 280	310, 315, 305, 275

Tc, tetracycline

[a] The amount of DNA used in each transformation was about 100 ng

[b] Rodriguez and Tait 1983

[c] Minimal medium was supplemented with all the required nutrients except for threonine

grow on minimal medium without threonine when they were subcultured in tetracycline-supplemented rich medium. Under these growth conditions, the recombinant cosmids were found to have lost part or all of their insert.

The two recombinant cosmids were isolated from Thr$^+$ clones and used to transform strain AB1133. Transformants were selected for Tc^r or for Thr$^+$. As shown in Table 2, fewer Tc^r transformants were obtained by use of Thr$^+$ recombinant cosmids than with the parent vector, for about the same amount of DNA (100 ng). The recombinant cosmids, if modified by the host modification system, were expected to be stable in these transformants. When replica-plated, all colonies from L + Tc plates (Rodriguez and Tait 1983) grew on minimal + Tc plates supplemented with all nutrients except threonine. Of 10 clones, taken at random from each set of plates, all were found to carry the appropriate Thr$^+$ recombinant cosmid. The sizes of DNA inserts for these recombinant cosmids, determined by digestion with various endonucleases, were estimated to be about 18 and 21 kb (data not shown).

To see whether *proA2*, a deletion mutation in *E. coli* K12 HB101, could be complemented by DNA fragments of *A. magnetotacticum*, another gene library containing larger fragment inserts was constructed. For this purpose, a similar cosmid, c2RB (Bates and Swift 1983), was used as the cloning vector. This cosmid is 6.8 kb long, contains two *cos* sites, and confers resistance to ampicillin. One of the advantages of this vector is the presence of a blunt end restriction enzyme site (*Sma*I) between the two *cos* sites that prevents cosmid concatamerization during ligation to the insert DNA. The insert DNA was prepared by partially digesting the DNA of *A. magnetotacticum* with *Sau*3A endonuclease and layering it onto a linear gradient of 5%–25% sucrose (Schwartz-Mann, ultrapure) prepared in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl. The gradients were centrifuged in an SW40 rotor (Beckman Instruments) at 20000 rpm and 10° C for 16–18 h. The gradients were fractionated by collecting 0.5 ml aliquots from the bottom. The size of the DNA present in each fraction was determined by agarose gel electrophoresis. Fractions with DNA fragments in the 35–45 kb range were pooled and concentrated by ethanol precipitation. The concentrated DNA was ligated with the two cosmid arms generated by digesting the vector DNA with *Bam*HI and *Sma*I endonucleases (Bates and Swift 1983). The ligated DNA was packaged (as described earlier) and used to transduce strain HB101. This strain was used as host because in addition to carrying the *ProA2* mutation, it lacks the *E. coli* K12 restriction system (Table 1). The size of the inserts, determined by gel electrophoresis of the cosmid DNA isolated from 10 clones picked at random, was between 29 and 43 kb.

Because *E. coli* HB101 is auxotrophic for proline and leucine, the library was tested for the ability of the clones to grow on minimal medium in the absence of proline or leucine. Of 768 clones tested, 3 were Leu$^-$ and 1 was Pro$^-$.

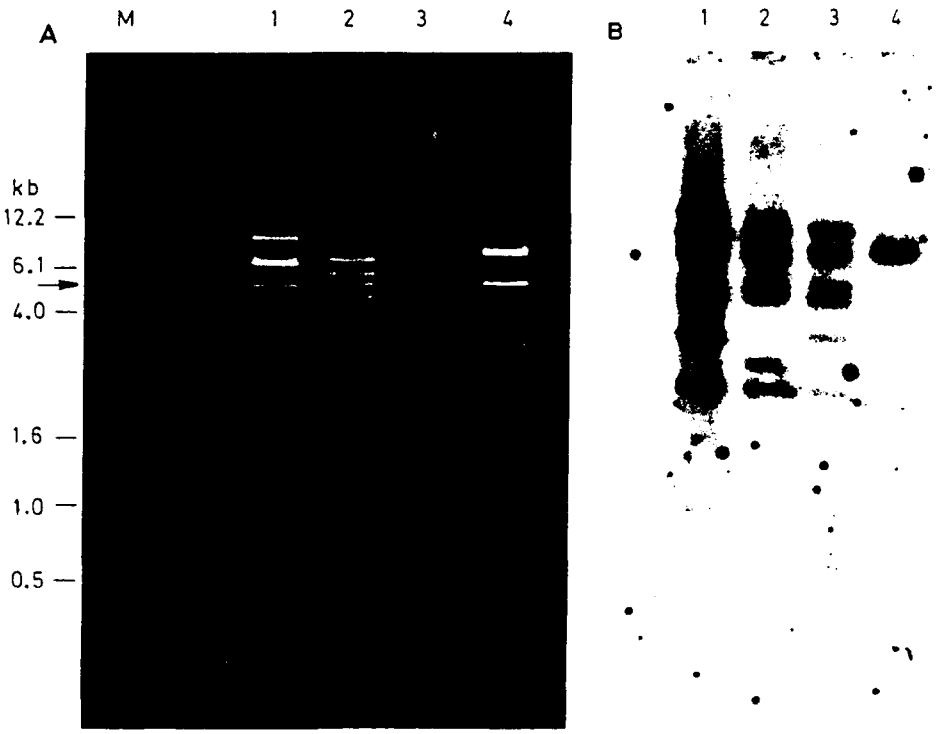

Fig. 1. **A** *Eco*RI digestion patterns of Leu$^-$ (lanes 1–3) and Pro$^-$ (lane 4) recombinant cosmids. The marker fragments, 1 kb ladder obtained from BRL, are shown in lane M. **B** Southern blot hybridization of Leu$^-$ and Pro$^-$ recombinant cosmids (shown in **A**) using the *Eco*RI-digested and ^{32}P-labelled DNA fragments of one of the Leu$^-$ recombinant cosmids (shown in lane 1) as probe. The vector present in the recombinant cosmids, (indicated by →) after the removal of the sequence between the two *cos* sites, is 1.7 kb smaller than the intact vector

All 4 recombinant cosmids were found to be stable in the host and maintained their appropriate phenotype after several rounds of subculturing in rich medium with ampicillin. The transformation of HB101 with each purified Leu$^+$ or Pro$^+$ recombinant cosmid yielded Ap^r colonies that were either 100% Leu$^-$ or Pro$^-$, respectively. In *E. coli* the *leu* and *pro* loci are far apart (Bachmann 1983). In *Desulfovibrio desulfuricans* Norway, another Gram-negative bacterium, the *leu* and *pro* biosynthetic genes are clusterd on the chromosome (Fons et al. 1987). In our study with *A. magnetotacticum*, none of the Leu$^+$ clones were Pro$^+$ or vice versa.

The sizes of the inserts, as determined by digestion with several endonucleases, were about 35, 39, 40, and 42 kb for the 3 Leu$^+$ and the 1 Pro$^+$ recombinant cosmids, respectively. The *Eco*RI digestion patterns of the recombinant cosmids is shown in Fig. 1a. *Eco*RI digestion separates the insert DNA from the vector DNA. Southern hybridization analysis of the *Eco*RI digests of the recombinant cosmids using one of the Leu$^+$ recombinant cosmids as probe (Fig. 1b) demonstrates that the 3 Leu$^+$ recombinant cosmids overlap by at least 22 to 25 kb (lanes 1–3). The DNA fragment inserts containing the Leu$^+$ and Pro$^+$ (lane 4) loci show no DNA homology with each other.

To confirm the phenotype of the Pro$^+$ recombinant cosmid and to further characterize its insert DNA, the cosmid was introduced into *E. coli* strains AD37, X340, and X342 with known *pro* mutations (Table 1). The proline biosynthetic pathway in *E. coli* involves four reactions, of which three are catalyzed by *proA*, *proB*, and *proC* gene products (Hayzer and Lensinger 1980; Csonka and Baich 1983). The *proA* and *proB* loci are linked and are located 3 min apart from the *proC* locus (Bachmann 1983). The complementation studies indicated that the Pro$^+$ recombinant cosmid restored wild-type phenotype in *proA*, *proB*, and *proAB* but not in the *proC* mutants. Under selective conditions of growth, only about 9 kb of the insert DNA was kept by the Pro$^+$ transformants of strain AD37 or X340. The 9-kb fragments isolated from several clones appeared to be identical on the basis of their digestion patterns with various endonucleases. Under non-selective growth conditions all 10 clones tested had lost their insert DNA (unpublished results).

The experiments described above demonstrate that DNA from *A. magnetotacticum* can be functionally expressed in *E. coli*. This finding is a crucial first step in developing a genetic analysis system for this organism that will permit us to study one of the most interesting properties of magnetotactic bacteria, i.e. the mechanism by which they acquire iron from the environment and assimilate it into single-domain magnetic particles.

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Cloning and characterization of the *recA* gene of *Aquaspirillum magnetotacticum**

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Abstract. The *recA* gene of *Aquaspirillum magnetotacticum* has been isolated from a genomic library and introduced into a *recA* mutant strain of *Escherichia coli* K12. The cloned gene complemented both the recombination and DNA repair deficiency of the host and its protein product promoted the proteolytic cleavage of the LexA protein. A protein whose molecular weight is similar to that of the RecA protein of *E. coli* was associated with the cloned sequence.

Key words: *RecA* gene – *Aquaspirillum magnetotacticum* – Gene library – Recombinant cosmid

Aquaspirillum magnetotacticum is a Gram-negative freshwater spirillum that synthesizes nanometer-sized, single-domain magnetic particles (for review see Blakemore 1982). We have recently constructed a gene library from the genomic material of this organism, which we have used to complement auxotrophic mutant strains of *Escherichia coli* K12 (Waleh 1988). To investigate whether mutations other than amino acid auxotrophy could be complemented with *A. magnetotacticum* genes, we screened the library for sequences that would complement the *recA* function of *E. coli* K12.

RecA-like sequences have been isolated from a number of bacterial species (West et al. 1983; Pierre and Paoletti 1983; Keener et al. 1984; Ohman et al. 1985; Goldberg and Mekanlaos 1986; Koomey and Falkow 1987). The *recA* gene product in *E. coli* is involved in homologous recombination (Clark 1973) and DNA repair (Hanawalt et al. 1979; Walker 1984). This protein also regulates the expression of a number of unlinked chromosomal genes by promoting the proteolytic cleavage of their repressor molecule, the LexA protein (Walker 1984). The LexA protein is also the repressor of the *recA* gene (Mount 1977).

In this paper, we report the cloning and characterization of the *recA* gene of *A. magnetotacticum*. Hybridization experiments indicate that homology exists between the *recA* sequences of *E. coli* and *A. magnetotacticum*, and complementation studies suggest that the two RecA proteins are functionally similar. A protein whose gel migration pattern is similar to the RecA protein of *E. coli* is produced when recombinant clones are treated with DNA-damaging agents.

* This paper is affectionately dedicated to Prof. John L. Ingraham

Offprint requests to: N. S. Waleh

Materials and methods

Bacteria and plasmids. *Aquaspirillum magnetotacticum* strain MS-1 was provided by BioMagnetech Corporation. Strain HB101, F⁻ *hsd20* (rB^- mB^-) *recA13 ara-14 proA2 leuB6 thi-1 lacY1 galK2 xyll mtll supE44 str* λ^-, was used to propagate the gene library. CL142 (K12-Row) was used as the colicin indicator strain (Ozeki et al. 1962). Plasmid pJC859 (provided by John Clark, University of California at Berkeley, Berkeley, California, USA) is a pBR322 derivative and carries the *Escherichia coli recA* gene.

Culture conditions. *A. magnetotacticum* strain MS-1 was grown according to the procedures described by Blakemore et al. (1979). *E. coli* cells were grown in LB liquid or LB agar medium. For the induction of RecA protein, cells were grown in M9 medium supplemented with 0.3% casamino acids and 0.2% thiamine. Ampicillin (amp) was added at the final concentration of 50 μg ml when required. MMS plates were prepared by spreading 200 μl of a 2% aqueous solution on the surface of LB plates.

Cloning the recA gene. A gene library from DNA of *A. magnetotacticum* was constructed in a broad host-range cosmid c2RB (Bates and Swift 1983) as described previously (Waleh 1988).

Purification of plasmids and DNA fragments. Plasmids were purified by the procedure of Rodriguez and Tait (1983). DNA fragments were purified from low-percentage SEAPLAQUE gels (FMC Corporation, Rockland, ME).

UV survival measurement. Cells were grown in LB-amp liquid medium at 37° C overnight. They were then pelleted by centrifugation and resuspended in an equal volume of TEN (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl) buffer. The cell suspension was serially diluted in the above buffer and 0.01 ml volumes of dilutions were spotted on LB agar-amp medium. Plates were placed at a distance of 82 cm from a 15-watt germicidal low-pressure mercury lamp (GE G8T5) and irradiated for the indicated time periods. Plates were wrapped in aluminium foil to prevent

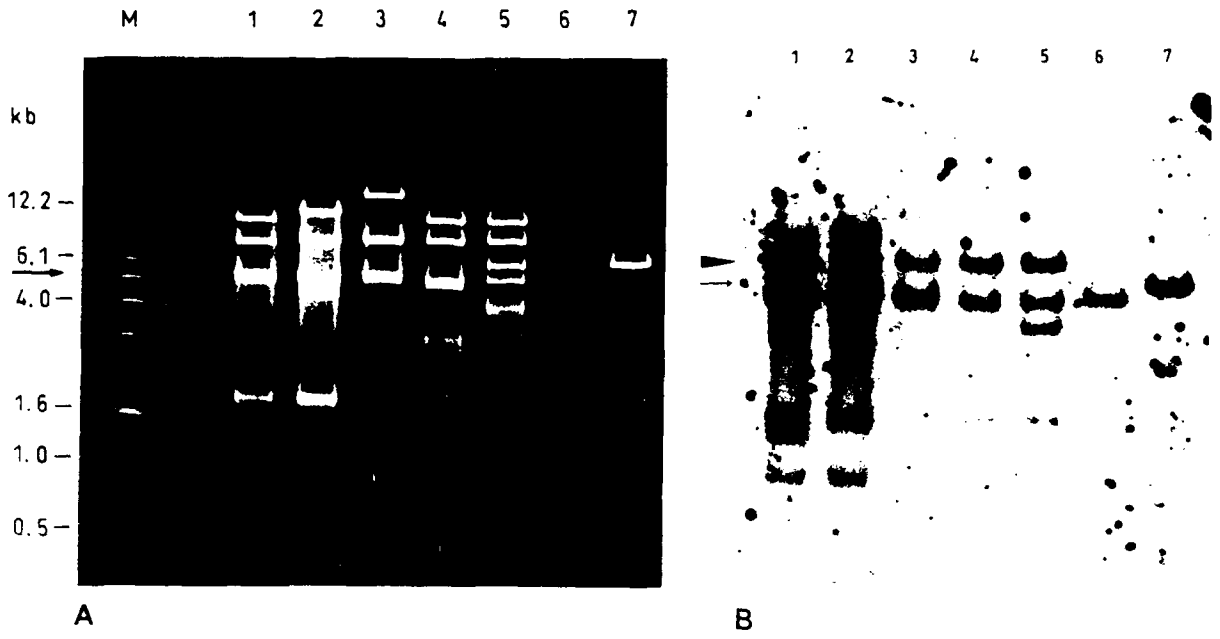

Fig. 1 A, B. *EcoRI* digestion patterns of RecA$^+$ recombinant cosmids (*lanes 1–5*), a control RecA recombinant cosmid (*lane 6*), and pJC859 (*lane 7*). The marker fragments, 1 kb ladder obtained from BRL, are shown in lane M. **B** Southern blot hybridization of DNA fragments shown in **A** using the *EcoRI* digested and ^{32}P-labeled DNA fragments of one of the RecA$^+$ recombinant cosmids (shown in *lane 1*) as probe. The vector band is indicated by → and the 8.0 kb *EcoRI* fragment shared by all RecA$^+$ recombinant cosmids is shown by ▶

photo-reactivation and incubated at 37 C overnight. The cross-streak method was used for screening a large number of colonies. In this test, overnight-grown cultures were streaked across LB agar-amp plates, and one-half of each streak was irradiated for 30 s. Plates were incubated at 37 C overnight in the dark. The RecA$^-$ mutant cells were killed at this UV dose, and the RecA$^+$ cells produced a thin film of growth in the irradiated parts of the streak.

Southern blot analysis. Plasmids were digested with *Eco*RI, electrophoresed in a 0.8% agarose gel, denatured *in situ*, and transferred to nitrocellulose filters as described by Maniatis et al. (1982). *Eco*RI-digested DNA fragments of recombinant cosmids were labeled with [γ^{32}P]ATP by T4 DNA kinase and were used as probe. The probe from *E. coli recA* gene was prepared by nick-translation using a BRL kit. The nitrocellulose filters were baked in an 80 C vacuum oven for 2 h and hybridized for 22 to 24 h at 45 C in a solution containing 50% formamide, 5 × SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.8% Denhardt's solution (Maniatis et al. 1982), and 300 μg of heat denatured salmon sperm DNA. Filters were washed at room temperature in 2 × SSC – 0.1% sodium dodecyl sulfate (SDS) for 20 min, at 45 C in 0.2 × SSC – 0.1% SDS for 30 min, wrapped in Saran wrap, and exposed to X-ray film at – 70 C using an intensifying screen (Cronex Hi-Plus).

Colicin test. Colonies were spotted on LB agar plates and were incubated at 37 C. After overnight incubation, cells were killed by exposure to chloroform vapor for 30 min and overlayed with 3 ml soft agar seeded with strain CL142. The colicin-producing colonies produced a zone of inhibition in the lawn of the indicator strain.

Induction of RecA protein. Cells were grown in minimal medium in a shaking 37 C incubator to an optical density of about 0.5 – 0.6 at 660 nm. At this time, cells were exposed either to UV light for the indicated periods of time or to mitomycin C added to the cultures at the final concentration of 1 μg ml. Cells were shaken at 37 C in the dark for two additional hours. Samples were taken at indicated times; cells were pelleted and stored at – 20 C.

Polyacrylamide gel electrophoresis. Cell pellets were resuspended in a dye mixture consisting of 1% SDS, 20% glycerol, 40 mM Tris-HCl, pH 6.8, 0.05% bromphenol blue (BPB), and 0.14 M 2-mercaptoethanol, boiled for 5 min in a boiling water bath, and electrophoresed through a 15% acrylamide gel. Cells were stained with Coomassie brilliant blue.

Results

Isolation of the Aquaspirillum magnetotacticum recA gene

The gene library prepared from chromosomal DNA of *A. magnetotacticum* was propagated in HB101, a *recA* mutant strain of *Escherichia coli* K12. This strain is sensitive to DNA-damaging agents, such as MMS, because of its deficiency in homologous recombination and DNA repair functions. Library clones that grow in the presence of MMS should therefore carry sequences that complement the *recA* deficiency and allow the growth of their host strain. Of 542 amp-resistant (amp^r) clones tested, we found 5 that grew in the presence of MMS. Plasmid analysis indicated that all clones carried recombinant cosmids with inserts that were between 26 – 35 kb in size. The *EcoRI* digestion patterns of the recombinant cosmids is shown in Fig. 1A. *EcoRI* digestion separates the insert DNA from the vector DNA. Southern hybridization analysis of the *EcoRI* digests of the recombinant cosmids using on the RecA$^+$ clones as probe

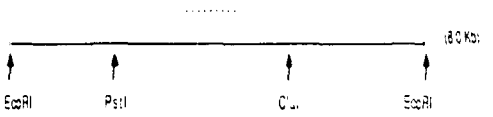

Fig. 2. Preliminary restriciton map of the 8.0 kb *EcoRI* fragment carrying the *recA* gene of *Aquaspirillum magnetotacticum*. The dashed line shows the approximate location of the *recA* gene as suggested by subcloning experiments described in the text

(Fig. 1 B) demonstrates that all five RecA$^+$ recombinant cosmids, in addition to the vector band, share a fragment of about 8 kb (lanes 1 – 5). As expected, no DNA homology (except for the cosmid DNA) was detected between the fragment inserts of the RecA$^+$ clones and those of an amp^r but MMS-sensitive (MMS^s) clone of the library (lane 6) that was used as negative control. *EcoRI* cleaves pJC859 into two fragments of 5750 and 1850 bp in size (Fig. 1 A, lane 7). In the hybridization experiment, however, only the larger fragment shows homology with the *recA* sequence of *A. magnetotacticum* (Fig. 1 B, lane 7). This fragment carries about 80% of the *E. coli recA* gene, including the promotor and the operator sequence (Sancar et al. 1980). When *E. coli* sequence was used as probe, only the 8-kb fragment of the RecA$^+$ recombinant cosmids, hybridized with the labeled probe (data not shown).

Restriction mapping of the A. magnetotacticum recA gene

The restriction digestion and Southern hybridization analysis described above indicated that all five RecA$^+$ recombinant cosmids shared a fragment of about 8 kb that hybridized with the labeled *E. coli recA* sequence. This fragment was purified and cloned into the *EcoRI* site of pBR322. The recombinant plasmid thus formed was used to transform HB101. All amp^r transformants were found to be MMS^r. When 20 of the amp^r MMS^r clones were tested for their sensitivity to UV light, all were found to be also UV resistant (UV^r). These results indicated that the 8-kb fragment carries the *recA* sequence of *A. magnetotacticum*.

The 8-kb fragment was digested with *PstI* and/or *ClaI* endonucleases and the resulting fragments were cloned into pBR322 digested with the same endonucleases. Upon screening of the transformants and identification of the fragments that conferred MMS^r and UV^r, the *recA* sequence of *A. magnetotacticum* was localized to a fragment of about 3-kb between *PstI* and *ClaI* restriction sites (Fig. 2).The 3-kb fragment was further purified and ligated with pBR322 digested with *PstI* and *ClaI* endonucleases. The ligated DNA molecules were used to transform HB101. Since *PstI* and *ClaI* digestions inactivate both antibiotic resistance markers of pBR322, transformants were selected for MMS^r and were further tested for UV^r. All MMS^r transformants were found to be UV^r and to carry the plasmid for the expected size. This plasmid construct was designated pNW300.

Complementation studies with A. magnetotacticum recA gene

The recombination proficiency of HB101 clones carrying the *recA* gene of *A. magnetotacticum* was determined by measuring the plating efficiency of a *red*$^-$ *gam*$^-$ mutant strain of bacteriophage λ (λ Fec$^-$ phenotype). This mutant phage requires the recombination activity of the RecA protein for its growth in *E. coli* cells (Manly et al. 1969). All

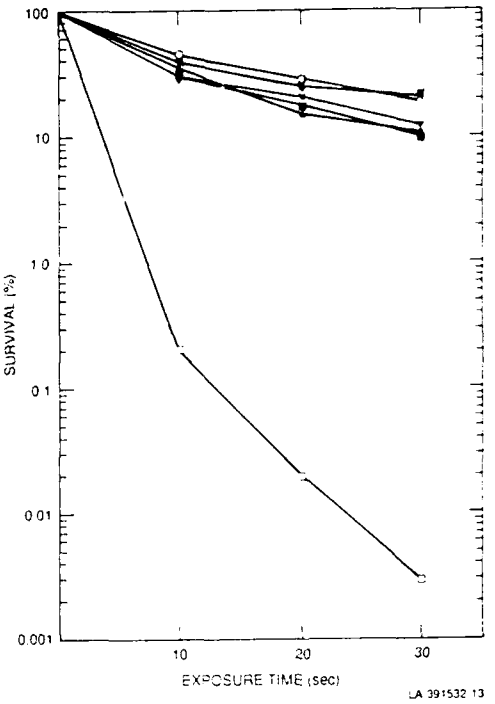

Fig. 3. UV survival of cells carrying the *recA* gene of *A. magnetotacticum*. Symbols: ×, ▲, ■, ▼, and ●, RecA$^+$ library clones; ○, HB101 (pJC859); and □, a RecA$^-$ library clone

clones carrying the *recA* gene of *A. magnetotacticum* supported the growth of λ *red*$^-$ *gam*$^-$ phage. The plating efficiency of the phage on these RecA$^+$ library clones was the same – about 30% of that obtained with strain HB101 (pJC859), which carries the *E. coli recA* gene. Strain HB101 alone did not support the growth of λ *red*$^-$ *gam*$^-$, and only few plaques were formed on plates with the lower dilutions of phage.

Because of their deficiency in DNA repair, the *recA* mutant strains of *E. coli* are sensitive to UV light. To see whether the *recA* gene of *A. magnetotacticum* can complement the mutant function, we examined the ability of the RecA$^+$ recombinant cosmids to repair the UV-damaged DNA of their *recA*$^-$ host. In these experiments, *E. coli* strain HB101, which carried pJC859, and a library clone picked at random were used as positive and negative controls, respectively. Quantitative UV survival measurements (Fig. 3) indicated that all recombinant cosmids with the cloned sequence conferred UV^r upon their host. The extent of protection in each case was similar to that confered by pJC859. No protection was detected by a control recombinant cosmid that was amp^r and MMS^s.

The RecA protein of *E. coli* promotes the proteolytic cleavage of the LexA protein which negatively regulates the expression of a number of unlinked chromosomal genes of *E. coli* (Walker 1984). Since LexA is also the colicin E1 gene repressor, we examined whether the RecA protein of *A. magnetotacticum* promotes the cleavage of the LexA protein and induces the expression of the colicin E1 gene. For this purpose, strains HB101, HB101 (pJC859) and HB101 (pNW300) were transformed with plasmid pNP12 (Waleh and Johnson 1985). This pBR322-derived plasmid confers resistance to tetracycline and carries the entire colicin E1 operon. Transformants were selected for tetracycline resis-

Table 1. Colicin production after induction of pNW300 and pJC859 with mitomycin C (MC)

Strains carrying plasmid pNP12	Colicin titer	
	− MC	+ MC
HB101 (pNW300)	0.01	1
HB101 (pJC859)	0.01	10
HB101	0	0

Results represent colicin titer $\times 10^{-2}$. The colicin titer is defined as the reciprocal of the last dilution giving noticeable clearing of the indicator lawn

tance and were tested for colicin production. Of 8 colonies tested, all pNP12-carrying clones of HB101 (pNW300) produced colicin. The zones of inhibition produced by these clones, however, were smaller than those produced by HB101 clones carrying plasmids pNP12 and pJC859 (6 mm versus 11 mm). As expected, none of the HB101 (pNP12) colonies produced any colicin. One transformant colony from each transformation set was picked and tested for the production of colicin in the presence of mitomycin C. Cultures, grown to midlog phase, were divided in half. To one half, mitomycin C was added at the final concentration of 1 μg ml; the other half was used as control. After 2 h of incubation, samples were taken, cells were pelleted, and the supernatants were titrated for colicin activity on a colicin-sensitive strain, CL142. The results (presented in Table 1) indicated that the amount of colicin produced by pNP12-carrying strain of HB101 (pNW300) was increased 100× upon treatment with mitomycin C. This amount of colicin was, however, tenfold less than the one produced by the pNP12-carrying strain of HB101 (pJC859) that carries the native gene. No colicin activity was detected in the HB101 (pNP12) culture supernatant.

Protein analysis of strain HB101 (pNW300)

Soluble protein extracts were prepared from untreated and mitomycin C-treated or UV-irradiated cells of HB101 (pNW300), HB101 (pJC859), and HB101; they were electrophoresed in a polyacrylamide gel and stained with Coomassie brilliant blue (Fig. 4). Treatment of HB101 (pNW300) with either mitomycin C or UV induced the production of a protein that migrated near the position of *E. coli* RecA protein. This protein was absent in extracts of strain HB101.

Discussion

We have cloned and partially characterized a DNA fragment of the genome of *Aquaspirillum magnetotacticum* that codes for a protein analogous to the *recA* gene product of *Escherichia coli* K12. The screening technique we used was based on heterologous complementation of an *E. coli recA* mutant that was first described by Better and Helinski (1983) for cloning the *recA* gene of *Rhizobium meliloti*. This technique was later used by others (Keener et al. 1984; Ohman et al. 1985; Goldberg and Mekalanos 1986; Koomy and Falkow 1987) to clone analogous *recA* sequences from other bacterial species.

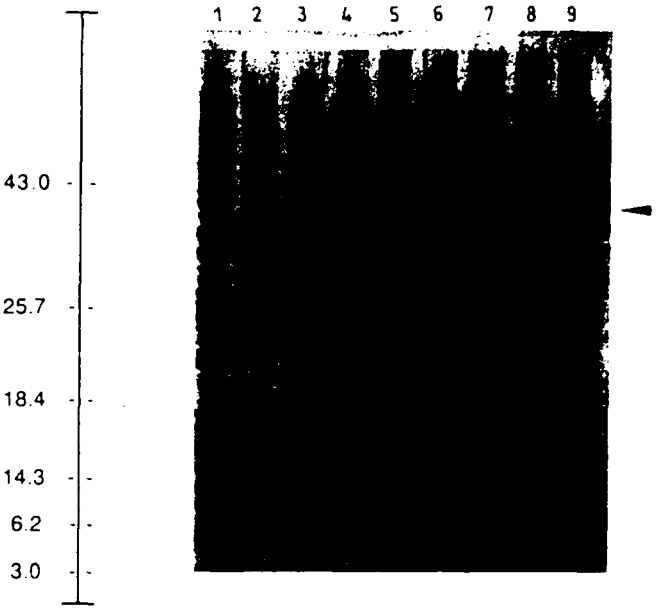

Fig. 4. Protein analysis of plasmid pNW300. Cells were grown in minimal medium to midlog phase when they were treated with mitomycin C at 1 μg ml, or UV irradiated for 30 s. After 2 h of incubation at 37° C, cells were pelleted and treated as described under Materials and methods. *Lanes 1–3, 4–6,* and *7–9* show protein samples from control, mitomycin C-treated, and UV-irradiated cells of HB101, HB101 (pNW300), and HB101 (pJC859), respectively. Molecular weight protein standards are given $\times 10^{-3}$

DNA hybridization experiments demonstrate that significant homology exists between the *recA* sequence of *A. magnetotacticum* and that of *E. coli* K12. This homology appears to be mainly at the amino-terminal portion of the two sequences, however, as evidenced by hybridization of probes complementary to the *recA* of *A. magnetotacticum* with the fragment that carried the amino-terminal and nearly 80% of the total *recA* sequence of *E. coli* (Fig. 1).

The RecA protein of *A. magnetotacticum* restored recombination proficiency in the *E. coli recA* mutant host. This was demonstrated by increased plating efficiency of the λ Fec⁻, which requires the *recA* function of the host for growth. The lower number of λ plaques observed with the RecA protein of *A. magnetotacticum* may be due to inefficient expression from the heterologous promoter or, alternatively, due to instability of the RecA protein in a foreign host. Indeed, in crude cell extracts, the RecA protein of *A. magnetotacticum* appears to be unstable and degrades rapidly upon short-term storage.

The RecA protein of *A. magnetotacticum* increased cell viability of the host to wild-type levels in response to UV exposure. HB101 cells with *recA* gene of *A. magnetotacticum* were as UV resistant as those carrying the native sequence. Similar levels of protection have been reported in heterologous complementation studies with the RecA proteins of *Proteus vulgaris, Shigella flexneri, Erwinia carotovora,* and *E. coli* B r (West et al. 1983; Keener et al. 1984). However, RecA protein of *R. meliloti* has only partially suppressed the UV sensitivity of an *E. coli recA* mutant (Better and Helinski 1983).

The most interesting results were the findings that the *recA* of *A. magnetotacticum* not only recognizes the LexA protein *E. coli* but that the *recA* itself may be regulated by

this repressor molecule. Our colicin induction experiments clearly demonstrate that the RecA of *A. magnetotacticum* promotes the cleavage of the LexA repressor, which leads to the derepression of the colicin E1 operon. Moreover, the finding that both UV and mitomycin C increased the level of the RecA protein of *A. magnetotacticum* – a response observed with *E. coli* RecA – strongly suggest that the cloned *recA* gene is regulated by the LexA protein of *E. coli*. Whether a LexA-like protein exists in the native host and whether it regulates the expression of the *recA* gene of *A. magnetotacticum* remains to be demonstrated.

Our results and those of the other investigators discussed above provide compelling evidence that the RecA protein is structurally and functionally preserved among Gram-negative bacteria. DNA and amino acid sequence analysis of RecA from various species should provide valuable information about the history of the evolution of this important multifunctional bacterial protein. DNA sequence analysis of the *recA* gene of *A. magnetotacticum* is currently in progress.

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Nucleotide sequence of *recA* gene of *Aquaspirillum magnetotacticum*

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Submitted December 20, 1989 EMBL accession no. X17371

We have determined the nucleotide sequence of the *recA* gene of *A. magnetotacticum*. The coding region has 1032 base pairs, specifying 344 amino acids. The deduced protein has a Mr of 36,750, which is consistent with our previously reported estimated value (1). In the 5′ non-coding region, there is a potential ribosome binding site (underlined) and an incomplete SOS box (boxed).

The nucleotide sequence shows 61.6% homology with the *recA* sequence of *E. coli* (2). The amino acid residues essential for the recombinase, protease and ATPase functions of the *E. coli* recA protein (3–5) are either conserved in *A. magnetotacticum* or are substituted with similar amino acids. Of 24 amino acid residues believed to be the ATP binding domain of the *E. coli* RecA protein (6), 15 are conserved in the *A. magnetotacticum* protein. For identical amino acids, the codons used by *A. magnetotacticum* were often found to be different from those used by *E. coli*.

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  D  G  T  P  V  A  E  .
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Cloning of a sequence of *Aquaspirillum magnetotacticum* that complements the *aroD* gene of *Escherichia coli*

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Summary

A 2 kb DNA fragment isolated from a cosmid library of *Aquaspirillum magnetotacticum* strain MS-1 complements the aromatic-metabolite requirements and iron-uptake deficiencies of *Escherichia coli* and *Salmonella typhimurium* strains that lack a functional *aroD* (biosynthetic dehydrodquinase) sequence. All recombinant cosmids selected for their *aroD* complementation property carry this sequence. No DNA sequence homology has, however, been detected by Southern hybridization between the cloned fragment and the *aroD* gene of *E. coli* or the *qa2* (catabolic dehydroquinase) gene of *Neurospora crassa*.

Introduction

Aquaspirillum magnetotacticum is a Gram-negative, bipolarly flagellated, freshwater spirillum which, in the presence of ferric quinate (an iron source), synthesizes intracellular, single-domain, magnetic particles (Blakemore, 1975). These particles are membrane-bound and arranged longitudinally along the long axis of the cell (Balkwill *et al.*, 1980).

Very little is known about the genetic structure or biosynthetic ability of *A. magnetotacticum*. Genetic study of this bacterium is difficult because *A. magnetotacticum* is fastidious and requires elaborate techniques for growth and maintenance. However, the growth of this bacterium in defined medium lacking tryptophan indicates the presence of an aromatic pathway leading to chorismic acid.

Using recombinant DNA techniques, we have initiated an investigation to study the most interesting property of *A. magnetotacticum*, namely the mechanism by which it acquires iron from the environment and assimilates it into single-domain magnetic particles. Our initial studies (Waleh, 1988; Berson *et al.*, 1989; 1990) indicate that the genes of *A. magnetotacticum* are functionally expressed in *Escherichia coli* and that the transcriptional and translational elements of the two organisms are compatible. We have constructed a cosmid library from the genomic material of *A. magnetotacticum* and screened it for the genes that complement known defects in the iron-uptake pathways of *E. coli*. In this study, we describe the cloning and characterization of a 2 kb DNA fragment that complements the *aroD* (biosynthetic dehydroquinase) function of *E. coli* and *Salmonella typhimurium* mutant strains. No sequence homology, however, has been detected between the cloned fragment and the *aroD* gene of *E. coli* (Kinghorn *et al.*, 1981; Duncan *et al.*, 1986) or the *qa2* (catabolic dehydroquinase) gene of *Neurospora crassa* (Giles *et al.*, 1985).

Results

Library screening

The library was prepared from the DNA of *A. magnetotacticum* in c2RB cosmid as described previously (Waleh, 1988). This library was screened for sequences that would complement known mutations in the iron-uptake genes of *E. coli*. In one such experiment, we screened the library for sequences that would complement the *aroD* function of *E. coli* CL451. The *aroD* gene of *E. coli* codes for the enzyme 3-dehydroquinase which catalyses the third step of the early common pathway for the biosynthesis of chorismic acid, precursor of aromatic amino acids and enterochelin (Stocker, 1986). Enterochelin, a phenolate siderophore, is synthesized by *E. coli* and many Gram-negative bacteria (Neilands, 1981).

The Aro$^+$ library clones were selected as described in the *Experimental procedures*. Of about 1600 library clones plated 13 grew on the selection medium. One of the Aro$^+$ colonies carrying a recombinant cosmid, designated J, was used for further investigation. Upon digestion with *Eco*RI and cloning of the generated fragments, the *Aro*$^+$ phenotype was found to be associated with a 2 kb fragment. This 2 kb fragment was isolated and subsequently cloned into plasmid pBR322. The constructed

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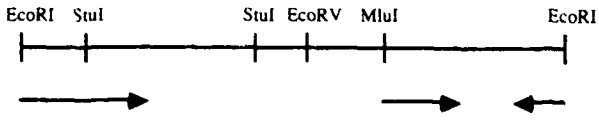

Fig. 1. Restriction map of the 2 kb fragment of *A. magnetotacticum*. The arrows indicate the sequenced regions.

plasmid conferred upon its *aroD* host the ability to grow on minimal medium without any aromatic amino acid supplements, and synthesized enterochelin as was demonstrated by its ability to remove iron from the medium.

The plasmid carrying the 2 kb fragment was then transferred into *E.coli* strain HB101 *fepA entA*. This strain is iron-uptake-deficient because it lacks the receptor (*fepA*) and one of the biosynthetic genes (*entA*) of enterochelin (De Lorenzo and Neilands, 1986). As expected, the plasmid did not mediate any siderophore synthesis in this host. However, it mediated growth of this strain in the presence of inhibitory concentrations of the chelating agent 2,2′-dipyridyl. Although we do not know whether the *aroD* activity and the chelating property are associated with one (or more) gene product, we hope to resolve this issue by sequencing the entire fragment. A restriction map and the sequenced regions of the 2 kb fragment (indicated by arrows) are presented in Fig. 1.

Complementation of the aroD *function of* S. typhimurium

The cloned 2 kb fragment was also tested for complementation of the *aroD* function of *S. typhimurium*. Plasmid pBR322, carrying the cloned fragment, was used to transform four *S. typhimurium* mutant strains with point mutations *aroD41*, *aroD68*, *aroD85* and *aroD88* (Nishioka *et al.*, 1967). To avoid restriction, the plasmid DNA was subjected to modification systems of *S. typhimurium* LB5000 prior to transformation. LB5000 is restriction-deficient but modification-proficient for all the three modification systems of *S. typhimurium* (Bullas and Ryu, 1983; Sanderson and Stocker, 1987.

The results indicated that the cloned fragment also complements the *aroD* function of *S. typhimurium*. The transformant colonies grew on minimal medium without aromatic metabolites and regained the ability to remove iron from the medium. The iron-uptake proficiency of the transformed *aroD41* and *aroD68* mutants was tested on the universal assay medium, as described in the *Experimental procedures.*

Southern blot analysis

The DNA homology of the cloned 2 kb fragment with the *aroD* gene of *E. coli* and *qa2* of *Neurospora crassa* was determined by Southern blot analysis. The *qa2* gene, which codes for catabolic 3-dehydroquinase, is a member of a gene cluster which codes for the five central steps of the fungal shikimate pathway (Giles *et al.*, 1985). The cloned *qa2* gene complements *E. coli aroD* mutants that lack biosynthetic 3-dehydroquinase activity (Schweizer *et al.*, 1981). The *qa3* gene, which codes for quinate dehydrogenase and is closely linked to *qa2*, was used as the negative control.

Plasmids carrying the *aroD* gene of *E. coli* and the *qa2* and *qa3* genes of *N. crassa* were digested with *Cla*I, *Hin*dII/*Bam*HI, and *Sal*I/*Bam*HI, to yield fragment inserts of about 1.8, 1.9, and 1.7 kb, respectively (Fig. 2A). As shown in Fig. 2B, no homology was detected between the cloned 2 kb fragment and the *aroD* or *qa2* sequences. No sequence homology was detected between the *aroD* gene of *E. coli* and the *qa2* of N. crassa.

Discussion

We have cloned a DNA fragment from the genome of *A. magnetotacticum* that complements the *aroD* function of *E. coli* or *S. typhimurium*. The cloned fragment does not mediate any siderophore synthesis in the iron-uptake-deficient strain, HB101 *fepA entA*, as determined by the chemical assay medium of Schwyn and Neilands (1987). However, it restores the ability of *E. coli* and *S. typhimurium aroD* mutants to remove iron from the medium, presumably by allowing them to synthesize the iron-binding compound, enterochelin.

Enteric bacteria use several siderophore-mediated systems for utilization of iron(III) in the medium. They synthesize the phenolate siderophore enterochelin and/or the hydroxamate siderophore, aerobactin (Braun and Winkelmann, 1987; Neilands, 1981). They also utilize some siderophores that they themselves do not synthesize (Braun and Winkelmann, 1987; Neilands, 1981). Mechanisms of iron uptake not involving any siderophore production have been demonstrated in *Serratia marcescens* (Zimmermann *et al.*, 1989) and in *Neisseria* species (West and Sparling, 1985). A siderophore of the hydroxamate type has been reported to be produced by *A. magnetotacticum* (Paoletti and Blakemore, 1986). Although the cloned 2 kb fragment described in this study restored the iron-uptake deficiencies of its *E. coli fepA entA* host, it did not mediate any siderophore synthesis. It is possible that *A. magnetotacticum*, like enteric bacteria, has multiple pathways for acquisition of iron from the environment. The importance of the cloned system with respect to iron transport and assimilation in *A. magnetotacticum* awaits further studies.

Although the cloned 2 kb fragment complements the *aroD* functions of both *E. coli* and *S. typhimurium* mutant strains, it shows no sequence homology with the *aroD*

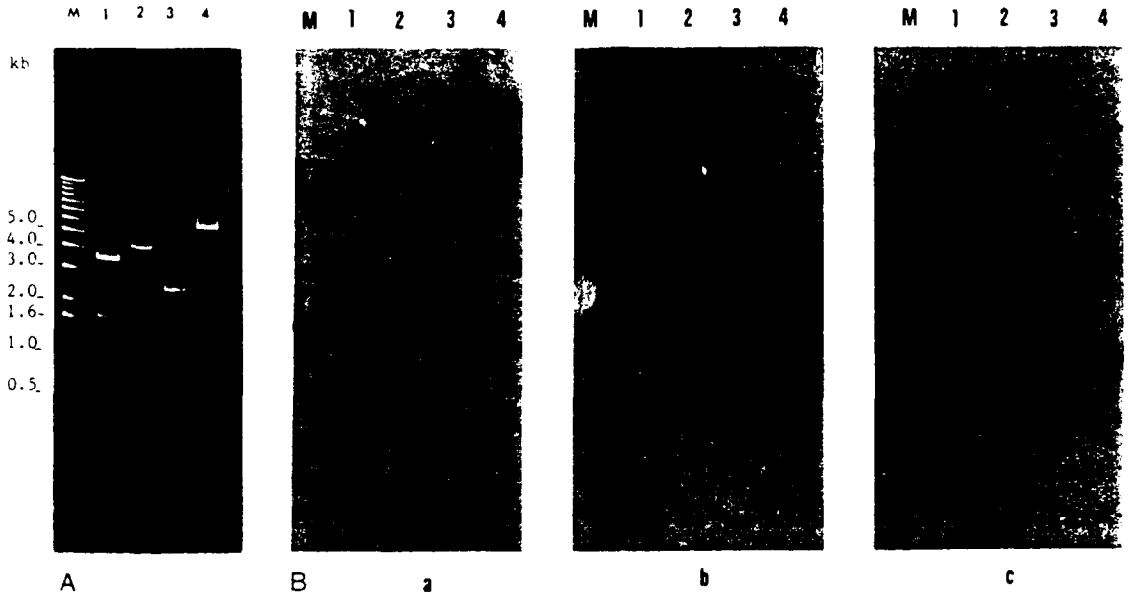

Fig. 2. Plasmids carrying the 2 kb fragment of *A. magnetotacticum*, the *aroD* gene of *E. coli*, or the *qa2* or *qa3* of *N. crassa* were digested with appropriate restriction endonucleases and electrophoresed in 0.7% agarose gel. The DNA fragments were blotted onto a nitrocellulose filter and hybridized with appropriate probes, as described in the text.
A. Lane M, 1 kb molecular-weight markers (BRL); lane 1, *E. coli aroD* gene (1.8 kb; lane 2, the cloned 2 kb fragment of *A. magnetotacticum*; lane 3, *qa2* gene of *N. crassa* (1.9 kb); lane 4, *qa3* gene of *N. crassa* (1.7 kb).
B. Blots prepared from the gel of Panel A hybridized with (a) the *E. coli aroD* gene; (b) 2 kb fragment of *A. magnetotacticum*; and (c) *qa2* of *N. crassa*.

sequence of *E. coli*. These findings are similar to those obtained with the *qa2* gene of *N. crassa*. The *qa2* gene complements the *aroD* function of *E. coli* (Schweizer *et al.*, 1981) but shows no homology with this gene either at DNA (this study) or amino acid sequence level (Duncan *et al.*, 1986). The *aroD*- and the *qa2*-coded enzymes are currently placed in separate classes of 3-dehydroquinases (Coggins and Boocock, 1986). The *aroD*-coded enzyme of *E. coli* is monofunctional (Berlyn and Giles, 1969; Chaudhuri *et al.*, 1986) and catalyses the third step in the common pathway for the biosynthesis of aromatic amino acids. The *qa* gene cluster of *N. crassa* specifies the inducible enzymes involved in the utilization of quinate or shikimate as carbon source (Greever *et al.*, 1989); one of these genes, *qa2*, specifies a catabolic dehydrogenase, catalysing the same reaction (but in reverse) as the product of *aroD* gene of *E. coli* and *S. typhimurium*. A third class of dehydroquinases occurs in some plants in which the enzyme is bifunctional and occurs with shikimate dehydrogenase (Coggins and Boocock, 1986). Comparison of the amino acid sequence of the *A. magnetotacticum* *'aroD–qa2'* gene product with other dehydroquinases will be vital for the proper classification of this enzyme. Nucleic acid sequence analysis of this gene is currently in progress.

Experimental procedures

Bacteria and plasmids

A. magnetotacticum was provided by the BioMagnetech Corporation. *E. coli* strain HB101 *fepA entA* (De Lorenzo and Neilands, 1986) was obtained from Dr J. B. Neilands (University of California, Berkely, USA). *E. coli* strain CL451 (=LE392 *aroD*25::Tn*10*), *S. typhimurium* strain LB5000 ($r^- m^+$) (Bullas and Ryu, 1983; Sanderson and Stocker, 1987) and *S. typhimurium* strains with mutations *aroD41*, *aroD68*, *aroD85*, and *aroD88* (Nishioka *et al.*, 1967) were obtained from Dr B. A. D. Stocker at Stanford University. Plasmid pKD201, carrying the entire *aroD* gene of *E. coli*, was provided by Dr J. R. Coggins (University of Glasgow, Scotland). Plasmids carrying the *qa2* and the *qa3* genes of *N. crassa* were provided by Dr N. Giles (University of Georgia, USA).

Media and growth conditions

A. magnetotacticum was grown in a defined medium according to the procedure described by Blakemore *et al.* (1979). The *E. coli* and *S. typhimurium* mutant strains were grown in LB-liquid or LB-agar medium. For the selection of Aro^+ colonies, cells were plated on M9 minimal medium supplemented with 0.2% thiamine and 0.3% vitamin assay casamino acids (Difco). The universal assay medium for iron-uptake studies was prepared as described by Schwyn and Neilands (1987). Iron-uptake-proficient clones produce an orange halo in the blue background of this medium.

Southern blot analysis

Plasmids were digested with restriction endonucleases and the fragments were separated by gel electrophoresis in 0.7% agarose. The DNA bands were then blotted onto a nitrocellulose filter (Schleicher & Schuell) and hybridized with appropriate probes. Probes were labelled with ^{32}P using a multiprime DNA-labelling system (Amersham). Hybridizations were carried out at 42°C in 50% formamide, 5× SSC, 5× Denhardt's solution, and 0.3 mg ml^{-1} salmon-sperm DNA. Filters were washed with 1× SSC/0.1% sodium dodecyl sulphate (SDS) twice at room temperature for 15 min and once at 47°C in 0.02× SSC, 0.1% SDS for 30 min. Filters were exposed to X-ray film at −70°C using an intensifying screen (Cronex Hi-Plus).

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