Cah. Biol. Mar. (1998) 39 : 305-308

Signal transduction and motility genes from the bacterial endosymbionts of *Riftia pachyptila*

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Introduction

The chemoautotrophic bacterial endosymbionts of the hydrothermal vent tube-worm, *Riftia pachyptila* Jones, 1981 are the apparent sole source of nutrition to their host. The adult tube-worms lack a mouth and digestive system (Jones, 1981) and are never found without symbionts. The central role of the symbionts in providing their host with fixed carbon and their intracellular location suggests a tight integration and coordination of function (Felbeck & Childress, 1988). The symbionts have thus far eluded all attempts at cultivation and it is yet unclear how the tube-worms acquire the distinct bacterial symbionts from the myriad of free-living prokaryotes in the vent environment.

In this specific and obligate association, we would expect to find coordinate regulation of specific "symbiotic" genes by host signals and a bacterial response similar to the induction of gene expression in other intracellular associations between bacteria and animal or plant hosts. Bacteria monitor and adapt to changes in their environment by one of two ways: by modifying the expression of particular genes through a two-component signal transduction system (reviewed in Hoch & Silhavy, 1995 and Parkinson et al., 1992), or by moving to a more favourable environment through a chemotaxis system. For the lack of cultivated symbionts, we have taken a molecular approach to identify functional two-component systems involved in bacterial sensing and components of the chemotaxis pathway including methyl-accepting chemotaxis receptors and the flagellar protein.

Materials and methods

Specimens of *Riftia pachyptila* were collected at the 13° North vent site of the East Pacific Rise, at a depth of 2600 meters, using the research submersible *Alvin* during the HERO cruise, April 1992. The bacterial symbionts were isolated from *R. pachyptila* trophosome tissue and purified on a Percoll gradient as previously described (Distel & Felbeck, 1988). High molecular weight DNA was prepared from the isolated symbionts by lysis and extraction in agarose plugs as previously described (Stein et al., 1996).

Primers for the polymerase chain reaction were designed by aligning conserved sequences of known histidine protein kinases, methyl-accepting chemotaxis proteins and flagellar genes from eubacteria.

A symbiont genomic library was constructed from high molecular weight symbiont DNA and the fosmid vector pFos1 and replicated onto nylon filters for heterologous probing (Hughes et al., 1997). The resultant 1500-member symbiont library was probed with random prime-labelled PCR products amplified from symbiont genomic DNA. The filters were hybridized according to standard protocol and exposed to autoradiographic film to indicate positive fosmid clones.

The identified histidine kinase gene was tested for function by complementing an *Escherichia coli* (Migula, 1895) sensor kinase mutant. DNA from the identified fosmid clone was partially digested and fragments were ligated into a series of expression vectors. Two *E. coli* strains, DZ225 and ANCC22 were transformed and the resulting recombinants screened for a heterologous sensor kinase capable of phenotypic suppression of the *E. coli* mutations as described previously (Hughes, et al., 1997). ß-galactosidase and alkaline phosphatase assays were performed to quantify the phenotypic suppression observed qualitatively.

By a complementation approach, the symbiont methyl-accepting chemotaxis proteins were tested for function. *R. pachyptila* symbiont DNA was randomly digested and the products subcloned into a multicopy vector. The

constructs were then moved into mutant *E. coli* strains, KO607 and HCB339, containing mutations or deletions in all four of the *E. coli* MCP genes rendering them non-chemotactic. The resulting recombinants were screened for restoring chemotaxis on soft-agar (0.3% agar) motility plates. Positive recombinants were scored for chemotactic motility as movement away from the centre of the plate. By the same method, the symbiont flagellar gene was expressed in motility mutant *E. coli* strains, CSH4 and JA11 and screened for motility on soft-agar motility plates.

The sequence for the sensor kinase, *rssA* and the response regulator, *rssB* are available from the GenBank database under accession number U93704.

Results

In order to identify key components of signal transduction and chemotaxis, a symbiont genomic library was constructed and PCR primers were designed by aligning conserved sequences of known genes of interest. Probing of the symbiont fosmid library identified 70 clones that hybridized to the putative sensor kinase amplification products. Approximately, eight of these clones contained unique sensor kinases. One was selected for sequence analysis, which revealed two open reading frames (ORFs). The first was designated *rssA* (for *Riftia* symbiont signal kinase) and the second was designated *rssB* (for *Riftia* symbiont signal regulator). A database search revealed significant sequence similarity between the two deduced protein sequences and members of two-component regulatory families. The *rssAB* operon appeared to contain a sensor kinase and two response regulators: one response regulator is fused to the sensor kinase gene, and the second encodes an independent protein (Fig. 1). While not the most common type of arrangement, there are at least six other two-component systems with this type, three of which showed the highest sequence homology to RssA and RssB.

Expression studies of the cloned sensor kinase gene, *rssA* (pDH80) revealed a protein capable of phenotypic suppression of a sensor kinase mutation in *E. coli* as screened for by a phenotypic reversion from Lac^- to Lac^+ of the *E. coli* strain, DZ225 (Hughes et al., 1997). This complementation was further confirmed by transforming pDH80 into ANCC22 which resulted in a $PhoA^+$ phenotype or positive alkaline phosphatase activity. The enzyme activity observed qualitatively was confirmed quantitatively by directly measuring the enzyme activity of the recombinants (Table 1). Both ß-galactosidase and alkaline phosphatase activities were significantly higher in the recombinant than in the vector controls (pIN-IIIA alone). Thus, *rssA* apparently encodes a functional protein capable of "crosstalking" via phosphoryl transfer to the receiver domain of *E. coli* sensor kinase mutants.

A similar method of PCR and screening of the symbiont genomic library was used to identify components of the chemotaxis pathway. The methyl-accepting chemotaxis protein (MCP) receptor is a major component of this pathway and is responsible for sensing changes in concentrations of chemoeffectors in the environment. A signal is then transduced through cytoplasmic components which interact directly with the flagellar motor. Approximately, 10 MCP's have been identified from the

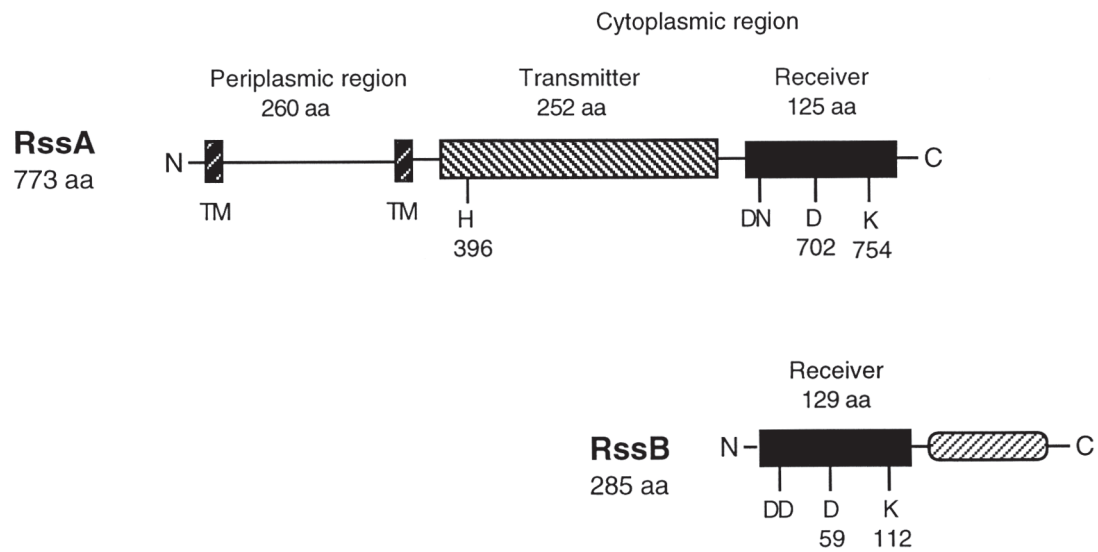

Figure 1. Domain organization of RssA/ RssB. The conserved histidine (H-396) of the sensor kinase domain in RssA and the aspartates (D-702, D-59) of the response regulator domain of RssA and RssB, respectively, are indicated. Putative membrane-spanning regions of the input domain are indicated by hatched bars. Modified from Hughes et al., 1997 *(Applied Environmental Microbiology,* **63**: 3494-3498).

Table 1. Quantification[a] of the phenotypic suppression observed by the pDH80 plasmid

Plasmid	Strain	ß-galactosidase (units)	alkaline phosphatase (units)
pDH80	DZ225	95.9±9 (n=3)	NA[b]
	ANCC22	NA	120±12 (n = 3)
pIN-IIIA[c]	DZ225	29±4 (n = 3)	NA
	ANCC22	NA	0.1±.003 (n = 3)

[a]enzyme activities were measured as described in Miller, J.H. 1972, *in Experiments in Molecular Genetics,* Cold Springs Harbor, 352-355. Values are means of three separate experiments.
[b]NA, not applicable.
[c]vector control
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symbiont genomic library suggesting that the symbionts of *Riftia* may possess as many as 10 chemotaxis receptors each sensing unique chemoattractants. One of the symbiont MCP genes was sequenced and found to contain all the necessary functional motifs of MCP's (Fig. 2) including the conserved methylation and signalling domains and the unique periplasmic ligand binding domain. Constructs were made with the identified MCP's and screened for their ability to complement *E. coli* chemotaxis mutants, KO607 and HCB339. Preliminary experiments suggests that at least one recombinant was found to restore chemotactic motility on soft-agar plates containing complex medium with further experiments in this area concentrating on identifying the specific chemoeffectors sensed by the recombinant MCP.

A similar approach of PCR and probing the symbiont library was used to identify a gene (*fliC*) which encodes the flagellar protein comprising the eubacterial flagellum. The predicted protein sequence of the symbiont FliC showed highest sequence homology to known flagellar proteins from enteric bacteria (>50% sequence identity through the conserved N and C-terminal portions of the protein). The symbiont flagellar gene was expressed in several motility mutant *E. coli* strains but was not found to restore motility to wild type levels. Further investigation using transmission electron microscopy revealed the presence of flagellated recombinant cells (unpublished data).

Discussion

Since its discovery 20 years ago, the symbiotic association of *Riftia* has been the subject of intense investigation. Fundamental questions of how the symbionts communicate with the host and their mode of transmission at each generation have long been pursued. Although the nature of the specific signals recognized by the symbionts is as yet unidentified, the existence of an apparently functional two-component system is the first indication that the symbionts possess a mechanism to communicate information about their external environment. Although flagella have not been detected in *Riftia* symbionts isolated from host tissue, the existence of functional chemotaxis and motility proteins suggests that the symbionts possess a free-living stage and may require the ability to be motile to colonize juvenile

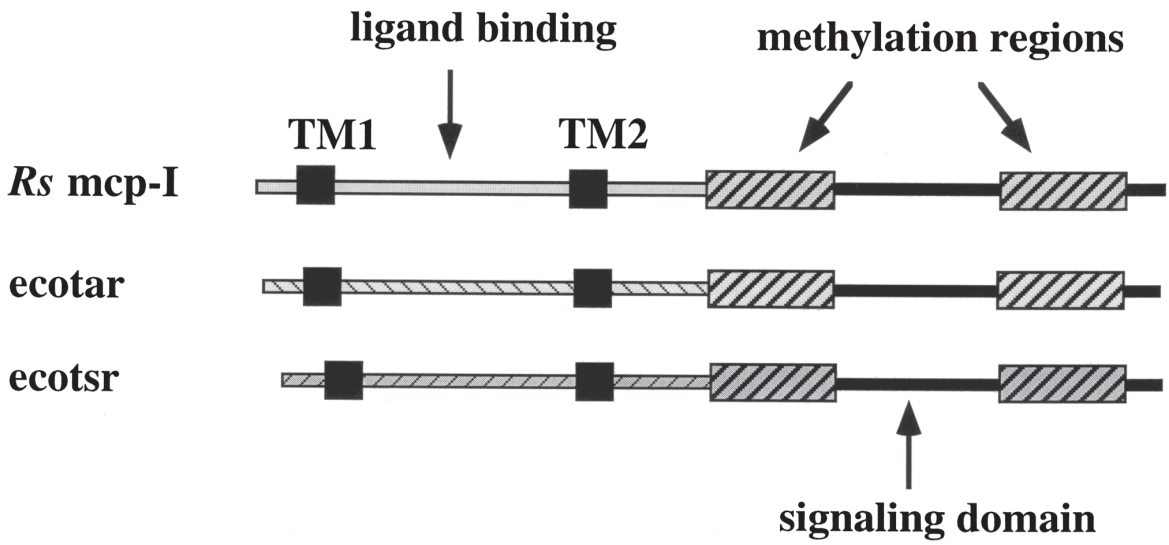

Figure 2. Predicted protein sequence alignment of the symbiont MCP, *Rs*mcp-I, as compared to two of the MCP's from *E. coli,* ecotar and ecotsr. The conserved cytoplasmic methylation and signaling domains are indicated as well as the two transmembrane domains and the unique periplasmic ligand binding domain of *Rs*mcp-I.

Riftia to establish a symbiosis within the host. Upon colonization, the flagella may be shed as it is in several other types of associations including the *Vibrio fisheri*/*Euprymna symbiosis* and the symbiosis of Rhizobia and leguminous plants. Motility is a requirement for these bacteria to be "symbiosis-competent" and to effect a successful colonization of the host organism. We can hypothesize a similar requirement for the symbionts of *Riftia pachyptila*. The results described here begin to address the question of how the symbiont and host communicate and effectively establish an intimate association and furthermore, provide the first direct evidence for a symbiont free-living stage.

Acknowledgements

The authors thank the captain and crew of the RV *Atlantis* II/ DSRV *Alvin*, and the RV *Nadir*/DSRV *Nautile* for their efforts during the 1994 and 1996 hydrothermal vent cruises.

This work was supported by National Science Foundation grants OCE93-17734 to J.L.S. and OCE93-14525 to H.F.

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