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Chemotaxis of *Leptospirillum ferrooxidans* and other acidophilic chemolithotrophs: comparison with the *Escherichia coli* chemosensory system

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1. SUMMARY

Ni^{2+}, Fe^{2+} and Cu^{2+} were attractants and aspartate was an apparent repellent for *Leptospirillum ferrooxidans*, a behaviour opposite to that for *Escherichia coli*. Membranes from *L. ferrooxidans* contained proteins with a molecular mass in the range of 80 kDa which were methylated in vitro. Methylation was stimulated in the presence of a membrane-free extract from *E. coli*, showing the response pattern expected for *L. ferrooxidans*, increased methylation by Ni^{2+}, and demethylation by aspartate. This suggests the existence of sensory transducers having a common methylation domain with the *E. coli* methyl-accepting chemotaxis proteins. Total chromosomal DNA digests from *L. ferrooxidans*, *Thiobacillus ferrooxidans* and *T. thiooxidans* hybridized with probes containing different domains of the *tar* gene from *E. coli*, implying the presence of *tar* type genes in the acidophilic bacteria studied.

2. INTRODUCTION

Motile bacteria are attracted by certain chemicals and repelled by others by means of a chemosensory system which regulates motility by controlling the direction of flagellar rotation [1–3]. Signals for a number of attractants and repellents are processed by structurally related sets of transmembrane proteins, known as transducers or methyl-accepting chemotaxis proteins (MCPs). These proteins receive the information from the environment and transduce that information into signals that converge on the flagellar motors [3,4]. For example, in *Escherichia coli* the Tar receptor is increasingly methylated in the presence of aspartate as attractant and is demethylated by Ni^{2+} and Co^{2+} as repellents [3].

Most of the industrially important microorganisms that participate in the bioleaching of miner-

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als, such as the chemolithotrophic acidophilic *Thiobacillus ferrooxidans, Leptospirillum ferrooxidans* and *T. thiooxidans* are motile by means of a single polar flagellum [5–8]. Therefore, they are expected to be chemotactic. The knowledge of this response is important in understanding the way these microorganisms sense and adapt to their environment. We have previously shown that *L. ferrooxidans* and *T. ferrooxidans* apparently possess sensory transducers for chemotaxis, since they responded chemotactically to Fe^{2+} and showed methylated proteins [9].

Although MCPs have been described for several heterotrophic microorganisms [10–16], the different proteins differ in their molecular masses. Despite this, there seems to have been a considerable conservation of the methylated regions among MCPs [11,15,17]. In the present report, we extend our previous results to confirm the presence of 80-kDa MCP-like proteins and DNA regions similar to the *E. coli tar* gene in chemolithotrophic acidophiles.

3. MATERIALS AND METHODS

3.1. Microorganisms and culture conditions

The chemolithotrophic bacteria used in this study were *T. ferrooxidans* ATCC 19859, *L. ferrooxidans* strains Z2 and *T. thiooxidans* ATCC 8085, all kindly supplied by Dr. A. Harrison. They were grown in Fe^{2+}-containing or sulfur-containing (*T. thiooxidans*) media as described before [9,18–20]. *E. coli* HB233 was kindly supplied by Dr. P. Engström and Dr. M. Nowlin and were grown as described before [1].

3.2. Chemotaxis capillary assay

The chemotaxis capillary assay [1] was used as before by employing radioactively labelled *L. ferrooxidans* obtained by growing the cells in the presence of $Na_2^{14}CO_3$ [9].

3.3. Preparation of cell-free extracts, crude membrane and high-speed supernatant fractions

The preparation of cell-free extracts after centrifugation at $7000 \times g$ was done as before [2,9]. Membranes obtained by disruption in the French cell at 12000 psi and the resulting soluble high-speed supernatant ($100000 \times g$ for 2–3 h) fractions from *E. coli* and *L. ferrooxidans* were essentially prepared as described by Foster et al. [21].

3.4. In vitro methylation of proteins

A total of about 200 μg of proteins (cell-free extract, membranes, high-speed supernatant, or a reconstituted homologous or heterologous mixture of the last two) and *S*-adenosyl [*methyl*-^{3}H]methionine (0.31 nmol, 80 Ci/mmol) were mixed, together with the various presumptive attractants or repellents (in a final volume of 40 μl of 50 mM Tris · HCl, pH 7.0, 0.1 mM EDTA), and incubated at 3°C for 20–60 min. The samples were then subjected to SDS-PAGE and fluorography [18,20].

3.5. DNA preparation, DNA probes and Southern blots

Chromosomal DNA from *E. coli, T. ferrooxidans, T. thiooxidans* and *L. ferrooxidans* was prepared according to the method of Marmur [22]. The plasmid pAK101 containing the *E. coli* chemotactic genes *cheA* (half of it), *cheW, tar* and a small segment of *tap* [23,24] was a kind gift of Dr. M. Simon. It was purified by CsCl buoyant density centrifugation [25].

We have chosen as a probe a 719-bp fragment located between nucleotides 763 and 1482 of the *tar* gene sequence [23,26]. This fragment corresponds to the most highly conserved cytoplasmic domain of the different chemotactic receptors from *E. coli* and other microorganisms [26]. It was amplified and labelled with digoxigenin by the polymerase chain reaction [27]. For this, we chemically synthesized the oligonucleotides 5′-TCACATATGCAACGCTCTTT-3′ and 5′-GGCAGCTGATTCCTGCACCA-3′ corresponding to nucleotides 763 to 782 and 1463 to 1482, respectively, from the *tar* gene sequence [23,26]. We also used as a probe a 782-bp fragment located between nucleotides −20 and 762 of the *tar* gene, containing the periplasmic domain and the transmembrane region of the receptor. In this case, the oligonucleotides were 5′-TGTTTTCAG-

GAAGTGCCTT-3′ and 5′-AACGCTCTGCGC-CAGGT-3′, corresponding to nucleotides −20 to 1 and 745 to 762 of the *tar* gene sequence [23,26]. In both cases the pAK101 plasmid was used as a template.

The DNA hybrids were detected by the non-radioactive enzyme-linked immunoassay procedure according to the manufacturer's instructions (Boehringer Mannheim). Southern blots [25] were carried out using Hybond-N blotting membranes from Amersham according to the manufacturer's recommendations. Hybridization was at 68°C.

4. RESULTS AND DISCUSSION

4.1. Metal ions are attractants for chemolithotrophs

By using the modified capillary assay [9], we found that not only Fe^{2+} but also Ni^{2+} and to some extent Cu^{2+} acted as attractants for *L. ferrooxidans* (Fig. 1). On the other hand, aspartate appeared to be a repellent, since the number of cells entering the capillary was even lower than the control. This behaviour was expected, since Fe^{2+} is oxidized by *L. ferrooxidans* to obtain energy and many organic compounds have been reported as inhibitory for these kind of bacteria [28]. Although Ni^{2+} is not oxidizable by *L. ferrooxidans*, it is not surprising that it behaved as an attractant, since non-metabolizable compounds have also been described as attractants in heterotrophic bacteria [29].

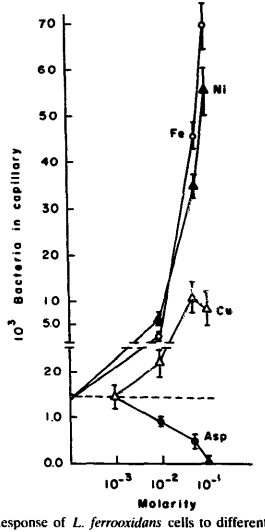

Fig. 1. Response of *L. ferrooxidans* cells to different chemotactic effectors. The number of radioactively labelled *L. ferrooxidans* cells entering the capillaries were measured at the indicated concentrations of Fe^{2+} (○); Ni^{2+} (▲); Cu^{2+} (△) and aspartate (●).

4.2. In vitro methylation of membrane proteins in response to chemotactic effectors

To study the proteins which are methylated in response to the effectors described above, we employed an in vitro methylation assay, since it is difficult to methyl-label in vivo the *L. ferrooxidans* proteins in the presence of [1-*methyl*-^{3}H]methionine and chloramphenicol. Figure 2 shows the in vitro methylation of a protein with a molecular mass in the region of 80 kDa in cell-free extracts from *L. ferrooxidans*. The maximal stimulation of the methylation of the 80-kDa protein was obtained at a final concentration of 50 mM Ni^{2+}. This protein may be similar to the methylatable transducers that have been described for other microorganisms. Although the molecular mass of the methylated *L. ferrooxidans* protein is higher than the approximate 60 kDa for the *E. coli* receptors, membrane chemotactic proteins with sizes in the range of 30–80 kDa have been detected in different eubacteria [12,13,16,30]. In the archaebacterium *Halobacterium halobium*, MCP-like proteins with a molecular mass between 90 and 135 have recently been described [10]. Since several regions of some of these proteins showed homology between them [23,26], it was interesting to check the effect of a cell-free extract from *E. coli* on the in vitro methylation of *L. ferrooxidans* membranes. Figure 3 shows that *E. coli* methylating enzymes stimulate protein methylation in *L. ferrooxidans* membranes (com-

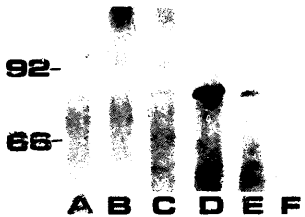

Fig. 2. Effect of Ni^{2+} on the in vitro methylation of proteins from *L. ferrooxidans*. Membrane-containing cell-free extracts from *L. ferrooxidans* were incubated in the presence of [^{3}H]AdoMet and without (A) or with Ni^{2+} 5 mN (B), 10 mM (C), 50 mM (D), 100 mN (E) and 300 mM (F) for 30 min at 37°C. The labelled components were analyzed by 10% SDS-PAGE and autoradiography.

pare lanes b and d). In addition, *L. ferrooxidans* membranes responded in the presence of *E. coli* enzymes as expected for the chemolithotrophic microorganism, i.e., stimulating methylation in the presence of Ni^{2+} (about 100% stimulation). A similar result was obtained when Fe^{2+} was used instead of Ni^{2+} (not shown). On the other hand, in the presence of aspartate and serine, there was an 80% and 50% inhibition of the methylation of the 80-kDa protein, respectively. These changes in the levels of methylation of the *L. ferrooxidans* 80-kDa membrane protein are in agreement with the chemotactic behaviour of the cells in the capillary assay (Fig. 1). In other words, the *L. ferrooxidans* putative chemotactic receptor would increase its methylation in the presence of attractants such as Ni^{2+} and would be demethylated in

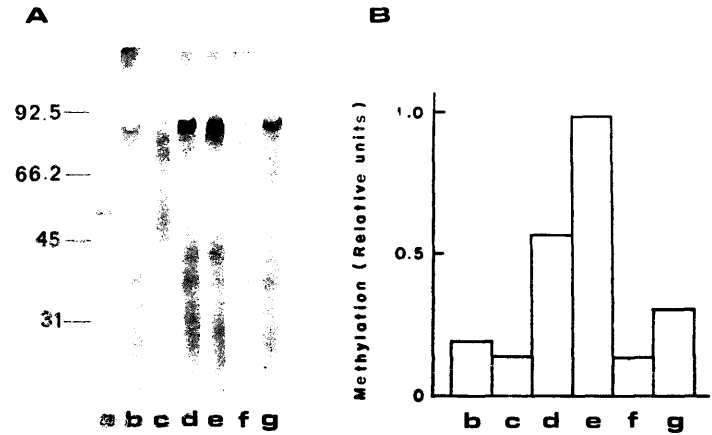

Fig. 3. Stimulation of methylation of *L. ferrooxidans* membrane proteins by *E. coli* enzymes. A. 130 μg of *E. coli* isolated membranes and 70 μg of high-speed supernatant of the same bacteria (a), 130 μg of *L. ferrooxidans* isolated membranes in the presence of 70 μg of proteins from high-speed supernatant from *L. ferrooxidans* (b), membranes alone (c) or in the presence of 70 μg of *E. coli* high-speed supernatant and no additions (d) or with 10 mN Ni^{2+} (e), 10 mM aspartate (f) or 10 mM serine (g) were all incubated in the presence of [^{3}H]AdoMet and were processed as in Fig. 2. B. The relative levels of methylation of the 80-kDa bands seen in A were determined by densitometry.

the presence of repellents such as aspartate. A result in agreement with the findings for *E. coli*, except that the attractants and repellents studied have opposite responses in both kinds of microorganisms.

4.3. Homology between the DNA from chemolithotrophic bacteria and the E. coli DNA coding for the Tar receptor

Our results support the notion that the MCPs have conserved methylated regions in a wide range of eubacteria [11,23]. This is not surprising since a chemotaxis-related methyltransferase enzyme from *B. subtilis* can recognize and methylate *E. coli* chemotactic membrane transducers [11]. To confirm this idea at the level of the genetic material, we employed as probes a DNA fragment containing the periplasmic domain and the membrane spanning region of the *E. coli* Tar protein (from the N-terminal end to amino acid 254) and a DNA segment including the methylated region and the most highly conserved region of the cytoplasmic domain of the Tar receptor (from amino acid 255 to 537). When the total genomic DNAs from *L. ferrooxidans, T. ferrooxidans* and *T. thiooxidans* were digested with restriction enzymes and the fragments separated and subjected to Southern blot analysis, we found the results shown in Fig. 4. Several restriction fragments hybridizing to the 782-bp fragment (periplasmic domain-containing) were evident in all the microorganisms tested. Also, we found a positive hybridization reaction with the 719-bp fragment codifying for the cytoplasmic and methylation region of the *E. coli* Tar receptor. When other restriction enzymes were used, simi-

Fig. 4. Analysis of chromosomal DNA from *L. ferrooxidans, T. ferrooxidans* and *T. thiooxidans* with probes coding for different domains of the *E. coli* Tar protein. Chromosomal DNA from *E. coli* (a, e), *L. ferrooxidans* (b, f), or *T. thiooxidans* (d, h) was completely digested with the restriction enzyme *Hin*dIII and the DNA from *T. ferrooxidans* (c, g) was digested with *Eco*RI. Fragmented DNA was ressolved by agarose gel electrophoresis and transferred to nylon membranes. Hybridization of the transferred DNA to the probe containing the periplasmic region (**A**) or the probe containing the cytoplasmic domain of the *E. coli* Tar receptor (**B**) was accomplished. Numbers in kilobases refer to lambda DNA markers obtained after digestion with *Ava*I.

lar results with different fragments were obtained (not shown).

These results indicate an homology between the domains of the *E. coli tar* gene, including the one which contains the methyl groups, and the DNA from the chemolithotrophic acidophiles studied. It will be of great interest to understand, at the level of the receptors, in which way an effector such as Ni^{2+} acts as an attractant for a chemolithotrophic microorganism and as a repellent for heterotrophs such as *E. coli*. To approach this, we are currently trying to isolate the *tar*-like gene from *L. ferrooxidans* to determine its structure in detail.

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