***Salmonella typhi* mutants defective in anaerobic respiration are impaired in their ability to replicate within epithelial cells**

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By using MudJ (Kan, *lac*)-directed operon fusion technology, mutants of *Salmonella typhi* whose gene expression is induced under anaerobic growth conditions were isolated. Characterization of their phenotypes and regulatory properties revealed that two of the mutants were unable to use nitrate as a terminal electron acceptor in the absence of oxygen, suggesting that they were defective in nitrate reductase activity. Anaerobic induction of these fusions did not further increase in response to nitrate. Strains carrying an additional mutation in *oxrA* were constructed. They showed a lower level of β -galactosidase expression both aerobically and anaerobically; however, the ratios of anaerobic induction remained unaltered. These MudJ insertions mapped to the 17–19 min region of the chromosome. Based upon their phenotypes and mapping, one of the mutants probably possessed a *modC* (*chID*)::MudJ insertion and the other a *moaA* (*chIA*)::MudJ insertion. A third mutant was unable to use either nitrate or fumarate as a terminal electron acceptor. All three mutants showed a reduced ability to enter into and proliferate within HEp-2 epithelial cells. The *oxrA* mutation enhanced entry and proliferation of both the wild-type cells and the three mutants. Taken together, these results suggest that anaerobic respiration plays a role in *S. typhi* invasiveness.

Keywords: *Salmonella typhi*, anaerobiosis, nitrate respiration, invasiveness

INTRODUCTION

Salmonella typhi, the causal agent of typhoid fever, is a specific human pathogen. Fundamental for *S. typhi* infectivity is its capacity to cross the mucosa of the distal ileum, as well as to survive and multiply within macrophages (Di Rita & Mekalanos, 1989; Finlay & Falkow, 1989; Groisman & Saier, 1990; Falkow *et al.*, 1992). To persist and establish a successful infection, *S. typhi* must be able to adapt to the fluctuating environmental conditions encountered within the human host. There is a growing body of evidence showing that bacterial pathogens are constantly sensing their environment and adjusting to it by regulating the expression of several genes and operons. This response often involves changes in the expression of genes encoding virulence factors (Miller *et al.*, 1989; Dorman, 1991; Falkow *et al.*, 1992; Mekalanos, 1992).

One of the conditions encountered by *S. typhi* during invasion of the intestinal epithelium, as well as in tissues during systemic infection of the human host, is low

oxygen availability (Lee & Falkow, 1990), for which enteric bacteria have developed adaptive responses. Facultative anaerobes, such as *Escherichia coli* and *Salmonella typhimurium*, can grow under aerobic or anaerobic conditions, deriving energy from a variety of respiratory or fermentative processes. Aerobically, oxygen serves as a terminal electron acceptor. Anaerobically, alternative respiratory chain acceptors, such as nitrate, nitrite, fumarate or dimethylsulphoxide, can be utilized (Poole & Ingledew, 1987; Lin & Kuritzkes, 1987). Both global and specific regulatory systems have been implicated in the regulation of anaerobic gene expression, to ensure that the most energetically favourable metabolic process is adopted (Spiro & Guest, 1991; Stewart, 1993; Guest, 1995).

There is evidence that oxygen availability may be an environmental signal controlling *Salmonella* virulence. It has been shown that anaerobiosis induces the invasion phenotype in *S. typhimurium* (Ernst *et al.*, 1990; Schiemann & Shope, 1991; Jones & Falkow, 1994) as well as in *S. choleraesuis* and *S. typhi* (Lee & Falkow,

1990; Tartera & Metcalf, 1993; Tartera *et al.*, 1993). Two oxygen-regulated invasion loci, *hil* (Lee *et al.*, 1992) and *orgA* (Jones & Falkow, 1994), required for *S. typhimurium* internalization into epithelial cells have been identified. However, their specific functions are unknown.

In a previous study we showed that the ability of *S. typhi* strain Ty2 to enter into and proliferate within HEp-2 epithelial cells was enhanced in anaerobically grown bacteria (Contreras *et al.*, 1995a). To identify anaerobically induced genes required for the invasion and proliferation of *S. typhi* strain Ty2 in cultured cells, we isolated several MudJ operon fusions in oxygen-regulated genes and characterized them as to their abilities to enter into and multiply within epithelial and phagocytic cell lines of human origin (Contreras *et al.*, 1995a). In the present work we further characterize three of these anaerobically induced fusions. These mutants are defective in the nitrate respiratory system and they are impaired in their ability to proliferate within HEp-2 cells. To our knowledge, the contribution of genes involved in anaerobic metabolism to the infectious capacity of facultative pathogens has not been reported previously.

METHODS

Bacterial strains, media and growth conditions. *S. typhimurium* strains MST1 (wild-type), TN3038 (*chlC::MudA*) and MST2970 (*oxrA2::Tn10 leuBCD485*) were obtained from S. Maloy, University of Illinois, Urbana, USA. *S. typhimurium* strain TT10269 (*chlC1130::Tn10*) was provided by J. Roth, The University of Utah, Salt Lake City, USA. *S. typhi* strain Ty2 (wild-type) was obtained from the Instituto de Salud Pública de Chile, Santiago, Chile, and *S. typhi* strain MCO01 (Ty2 MudJ Mucts MudJ) was obtained in our laboratory (Contreras *et al.*, 1994). The *S. typhi* insertion mutants described in this study and their properties are shown in Table 1. The complex medium used was Luria-Bertani broth (LB: Bacto tryptone, 10 g l⁻¹; Bacto yeast extract, 5 g l⁻¹; NaCl, 5 g l⁻¹). The minimal medium was medium E (MgSO₄·7H₂O, 0·02 g l⁻¹; citric acid, H₂O, 2 g l⁻¹; Na₂HPO₄·3H₂O, 13·1 g l⁻¹; NaNH₄HPO₄·4H₂O, 3·3 g l⁻¹) supplemented with cysteine (50 mg l⁻¹) and tryptophan (50 mg l⁻¹). Carbon sources were added to a final concentration of 0·2% (w/v). Where indicated, sodium nitrate or sodium fumarate were added to a final concentration of 20 mM. Dimethylsulphoxide was added to a final concentration of 0·1% (w/v). Solid media contained 1·5% (w/v) Bacto agar (Difco). When necessary, media were supplemented with kanamycin (50 µg ml⁻¹). Chlorate resistance was assayed by scoring growth on medium E agar plates containing glucose, sodium nitrate and potassium chlorate (15 mM) after incubation for 48 h under anaerobic conditions. Anaerobiosis was achieved by the use of GasPak anaerobic jars. Aerobic cultures (5 ml) were incubated in 125 ml flasks with vigorous shaking. Strains were grown at 37 °C.

Isolation of oxygen-regulated operon fusions. Random MudJ insertions in the *S. typhi* strain Ty2 chromosome were obtained as previously described (Contreras *et al.*, 1994). Briefly, a phage Mu lysate, obtained by heat induction (Bukhari & Ljungquist, 1977), of *S. typhi* strain MCO01 was used to infect *S. typhi* strain Ty2 at an m.o.i of 0·01 p.f.u. per

bacterium. After incubation for 20 min to allow phage adsorption followed by another 40 min with shaking in the presence of 15 mM EDTA, transductants were plated on LB agar plates containing kanamycin. After incubation for 18 h at 37 °C, colonies were replicated on LB agar plates containing kanamycin and the chromogenic substrate for β -galactosidase, X-Gal (Miller, 1972). Duplicate plates were incubated aerobically and anaerobically. After anaerobic growth the plates were placed at 4 °C under aerobic conditions for 30 min to allow oxidation of indole produced by hydrolysis of X-Gal during growth. Colonies that appeared to produce more β -galactosidase under anaerobic conditions (dark blue on X-Gal) were selected for further studies. β -Galactosidase assays were performed *in vitro* as described by Miller (1972) on cultures grown under aerobic and anaerobic conditions to mid-exponential phase (OD₆₀₀ = 0·2).

Clones regulated by oxygen availability were characterized as follows. The outer-membrane-enriched fraction was obtained as described by Contreras *et al.* (1995b) and proteins were analysed by SDS-PAGE in 12·5% polyacrylamide slabs using the procedure of Laemmli (1970) as modified by Lobos & Mora (1991). Motility was examined by stabbing into semi-solid MIO medium (Difco).

Genetic techniques. Transductions were performed by using the high-transducing phage P22 HTint as described by Maloy (1990). The approximate chromosomal location of MudJ insertions was determined by the method of Benson & Goldman (1992). This mapping technique is based on a set of *S. typhimurium* strains carrying locked-in Mud-P22 prophages in different locations on their chromosome. To use this technique, the MudJ insertions were first moved to *S. typhimurium* strain MST1 by transformation with linear fragments of chromosomal DNA from the mutants. The method used was based on the one described by Russell *et al.* (1989) and modified by N. Bossi & L. Bossi (Centre de Génétique Moléculaire, CNRS, France; personal communication). Electroporation was performed as described by O'Callaghan & Charbit (1990).

Invasion and intracellular proliferation assays. To measure invasion and intracellular proliferation of *S. typhi* strain Ty2 and its derivatives with oxygen-regulated operon fusions in HEp-2 epithelial cells, an *in vitro* assay based on the method described by Lissner *et al.* (1983) was used. Approximately 5×10^6 c.f.u. (100 µl) bacteria grown to mid-exponential phase were centrifuged, resuspended in 100 µl DMEMFS (Dulbecco's modified Eagle medium supplemented with 10%, v/v, foetal bovine serum) and added to six wells containing cell monolayers at a ratio of 10:100 bacteria per cell. After incubation for 1 h in a 10% CO₂/90% air atmosphere to allow for bacterial entry into the cells, monolayers were washed twice with phosphate-buffered saline (PBS: NaCl, 0·8%, w/v; KCl, 0·02%, w/v; Na₂HPO₄·2H₂O, 0·13%, w/v; KH₂PO₄, 0·02%, w/v), and then 100 µl DMEMFS containing gentamicin (250 µg ml⁻¹) was added to each well and the plates were incubated for 2 h to kill any remaining extracellular bacteria. The medium was removed and the cells were washed twice with PBS. Fresh medium containing gentamicin (25 µg ml⁻¹) was added and cells from three wells were lysed with sodium deoxycholate (0·5%, w/v, in PBS). The number of intracellular bacteria (c.f.u. at t_0) was determined by plating onto LB agar plates. The remaining three wells were incubated for 20 h to allow intracellular proliferation. The monolayers were then lysed and bacteria were counted (c.f.u. at t_{20}). Quantitative invasion assay results were calculated as follows:

$$\text{Invasion index} = \frac{100 \times (\text{c.f.u. intracellular at } t_0 / \text{c.f.u. added}) \text{ mutant}}{(\text{c.f.u. intracellular at } t_0 / \text{c.f.u. added}) \text{ parental}}$$

Proliferation index =

$$\frac{100 \times (\text{c.f.u. intracellular at } t_{20} / \text{c.f.u. intracellular at } t_0) \text{ mutant}}{(\text{c.f.u. intracellular at } t_{20} / \text{c.f.u. intracellular at } t_0) \text{ parental}}$$

RESULTS

Fusions in genes involved in anaerobic respiration are required for *S. typhi* proliferation within epithelial cells

From a collection of 12346 random MudJ insertion mutants of *S. typhi* we had previously selected 25 mutants which were shown, by β -galactosidase measurements *in vitro*, to contain fusions in genes whose expression was induced during anaerobic growth (Contreras *et al.*, 1995a). Three of the mutants, which were found to be impaired in invasiveness and proliferation within HEp-2 cells (Table 1), are further characterized in this work.

We confirmed that the impaired abilities to invade epithelial cells exhibited by the mutants were linked to

Table 1. Anaerobic induction of β -galactosidase activity and interactions of *S. typhi* mutants with HEp-2 cells

Strain	Invasion index*	Proliferation index*	β -Galactosidase activity†		
			+O ₂	-O ₂	-O ₂ /+O ₂
Ty2	100	100	0	0	—
DD46	3	10	363	1968	5.4
JJ3	66	47	477	1841	3.9
NN19	37	26	1201	2520	2.1

*Bacteria were grown under anaerobic conditions prior to assaying with the cultured cells.

† β -Galactosidase activity is expressed in Miller units (Miller, 1972). Data are from one representative experiment. Each experiment was repeated at least three times.

MudJ and were not due to a secondary mutation by backcrossing each MudJ insertion into a clean *S. typhi* strain Ty2 background and selecting for the Kan^r and X-Gal⁺ phenotype. Transformants showed an invasion-defective phenotype and anaerobic induction of β -galactosidase activity similar to the original MudJ insertion mutant (data not shown). Based upon Southern blot analysis, all the mutants described in this study contain single MudJ chromosomal insertions (Contreras *et al.*, 1994).

To investigate whether the fusions in mutants JJ3, NN19 and DD46 occurred in genes involved in anaerobic metabolism, the effect of sugars and alternative electron acceptors was studied. When the mutants were grown in E medium supplemented with glucose, generation times were similar to those in complex media (data not shown). The β -galactosidase levels of the mutants were similar under each of the anaerobic growth conditions tested (Table 2). Mutants JJ3 and NN19 were unable to grow in E medium supplemented with glycerol and nitrate. Because *S. typhi* cannot ferment glycerol anaerobically, growth in this medium must be sustained from nitrate respiration. These results suggest that these mutants lack nitrate reductase activity. Mutant DD46 did not grow in E medium supplemented with glycerol and either nitrate or fumarate as the terminal electron acceptor (Table 2). In addition, we observed that the three mutants, as well as the wild-type strain, were unable to grow in E medium supplemented with glycerol and dimethylsulphoxide, suggesting that *S. typhi*, in contrast to *S. typhimurium* and *E. coli*, lacks dimethylsulphoxide reductase activity.

Mutants defective in anaerobic respiration are chlorate resistant

To confirm that mutants JJ3, NN19 and DD46 were defective in nitrate reductase activity they were examined for their response to chlorate. Chlorate is reduced by nitrate reductase to yield a toxic product. Therefore, those mutants that are deficient in nitrate reductase activity are chlorate resistant under anaerobic conditions (Stewart & MacGregor, 1982; Stewart,

Table 2. Effect of culture medium on β -galactosidase activity of anaerobically induced operon fusions

Values are expressed as β -galactosidase activity in Miller units (Miller, 1972). Cultures were grown under anaerobic conditions in LB or E medium with the supplements indicated. Data are from one representative experiment. Each experiment was repeated at least three times.

Strain	LB	E + glucose	E + glucose + nitrate	E + glucose + fumarate	E + glycerol + nitrate	E + glycerol + fumarate
DD46	1626	1858	2773	2146	NG	NG
JJ3	1803	2215	3304	2596	NG	2584
NN19	2069	2422	2288	1878	NG	2045

NG, No growth.

Table 3. Chlorate resistance of *S. typhi* strain Ty2 and anaerobically induced fusions

Cultures grown in E medium supplemented with glucose, cysteine and tryptophan were diluted and plated onto E agar plates with glucose, cysteine and tryptophan (*) or with glucose, cysteine and tryptophan, supplemented with nitrate and chlorate (†). Plates were incubated under anaerobic conditions for 48 h.

Strain	C.f.u. without chlorate*	C.f.u. with chlorate†	Percentage survival
Ty2	1.5×10^8	3.9×10^3	0.003
DD46	3.4×10^9	2.2×10^8	6.5
JJ3	2.7×10^9	3.0×10^8	11.0
NN19	3.9×10^9	8.0×10^6	0.2

1988). As shown in Table 3, the mutants were 10^2 – 10^3 times more resistant to chlorate than the parental strain.

Lack of nitrate reductase activity leading to chlorate resistance is often caused by mutations in *chl* (*mol*) loci, which are implicated in the synthesis, insertion or processing of molybdenum cofactor (Stewart & MacGregor, 1982; Stewart, 1988). Therefore, we tested the effect of molybdenum on the growth of the mutants on glycerol and nitrate medium. Two of the mutants were unaffected by molybdenum, but growth of mutant NN19 was restored when cultured in E medium with glycerol and nitrate supplemented with sodium molybdate, the doubling time being similar to that of the parental strain (Fig. 1). This result suggests that the fusion in mutant NN19 could be in the *modC* (*chlD*) gene, involved in the active transport of molybdenum (Johann & Hinton, 1987; Miller *et al.*, 1987; Scott & Amy, 1989). In *E. coli*, *mod* mutants are unique in that the effect of the mutation can be suppressed by an increase in the molybdate concentration in the medium (Glaser & DeMoss, 1971; Sperl & DeMoss, 1975; Stewart & MacGregor, 1982).

MudJ insertions map to 17–19 min and 67–69 min regions of *S. typhimurium* chromosome

It has been previously reported that mutations in the *E. coli* nitrate reductase structural genes (the *chlC* locus or *narG* operon) do not result in a chlorate-resistant phenotype (Glaser & DeMoss, 1971; Stewart & MacGregor, 1982). Accordingly, when we transduced each mutant strain with a P22 lysate propagated on *S. typhimurium* strain TT10269 (*chlC*::Tn10) no replacement of the Kan^r phenotype with a Tet^r phenotype was found, indicating that MudJ fusions were not within the *chlC* locus.

The approximate chromosomal location of MudJ insertions was determined with the aid of strains carrying Mud-P22 insertions at defined positions on the *S. typhimurium* chromosome. The MudJ insertions mapped to the 17–19 min region (mutants NN19 and

JJ3) and 67–69 min region (mutant DD46) of the *S. typhimurium* chromosome.

The *oxrA* mutation affects expression of β -galactosidase from operon fusions

The *S. typhimurium* *oxrA* (*fnr*) gene product is a positive regulator of the anaerobically inducible genes, similar to the *E. coli* Fnr protein (Strauch *et al.*, 1985). To determine the effect of an *oxrA* mutation on the fusions studied in this work, we transduced an *oxrA2*::Tn10 insertion from *S. typhimurium* strain MST2970 into each mutant. In the case of mutants JJ3 and NN19, the *oxrA* mutation reduced expression of β -galactosidase under both aerobic and anaerobic conditions. However, the anaerobic induction ratio remained almost unaffected (Table 4), suggesting that other regulatory gene(s) are functional in *S. typhi*. The *oxrA* mutation clearly affected expression of β -galactosidase from mutant DD46, reducing the ratio of anaerobic induction to less than twofold (Table 4). The *oxrA* mutation had no additional effect on expression of the three fusions when the mutants were grown anaerobically in nitrate-supplemented media; the β -galactosidase levels remained similar to those measured in media without nitrate under anaerobic conditions (Table 4).

To determine the regulatory effect of the *oxrA* gene on the expression of the *chlC* gene in *S. typhi*, a strain carrying an *oxrA* mutation and a *chlC*::MudA fusion was constructed. As shown in Table 4, the *oxrA* mutation reduced expression of β -galactosidase under both aerobic and anaerobic conditions. However, the anaerobic induction ratio was enhanced about twofold (Table 4).

The *oxrA* mutation suppresses the invasion/proliferation defect of the anaerobically induced mutants

To evaluate the functional consequences of the *oxrA* mutation, we assayed the mutants ability to enter into and proliferate within HEP-2 cells. Unexpectedly, the *oxrA* mutation restored the capacity of the anaerobically induced mutants to invade epithelial cells (Table 5). Furthermore, our results suggest a potentiation of the invasiveness of the mutants. This effect was also observed with an *oxrA* derivative of the wild-type strain. These results indicate that the *oxrA* gene enhances both invasion and proliferation of *S. typhi*, and that these effects are independent of the three mutations characterized in this study. The reasons for the effect of the *oxrA* gene are currently being investigated.

Anaerobic growth induces synthesis of membrane proteins

It has been reported that chlorate-resistant mutants of *E. coli* have a complex phenotype (Graham *et al.*, 1980). The pleiotropic nature of such mutants has been proposed to result from alterations in the structure of the membrane (MacGregor & Schnaitman, 1971).

Fig. 1. Effect of the addition of molybdate on the anaerobic growth of *S. typhi* strain Ty2 (○) and chlorate-resistant mutants DD46 (▲), JJ3 (●) and NN19 (△). Bacteria were grown in minimal E medium supplemented with glycerol and NaNO₃ as described in Methods. (a) No additions; (b) with the addition of 1 mM Na₂MoO₄.

Electrophoretic analysis of membrane fractions showed that anaerobic growth strongly induced the synthesis of two proteins with apparent molecular masses of 41 and 28.5 kDa in the wild-type strain. On the other hand, a protein of 39.7 kDa was repressed (Fig. 2, lane C). The addition of nitrate to the growth medium caused the induction of a 134.8 kDa protein (Fig. 2, lane D). The latter protein band appeared in the membrane fraction of mutants DD46 and JJ3 when grown anaerobically, both in the absence and in the presence of nitrate (Fig. 2, lanes I, J, O and P), but it was not synthesized by mutant NN19 under any growth condition tested (Fig. 2, lanes K–M). In all the mutants a protein band with an apparent molecular mass of 118.5 kDa was visible in the

Table 5. Effect of an *oxrA* mutation on invasion and proliferation of *S. typhi* strain Ty2 and anaerobically induced fusions

Data are from one representative experiment. Each experiment was done in triplicate and repeated at least three (*) or six (†) times.

Strain	Invasion index	Proliferation index
Ty2	100	100
Ty2 <i>oxrA</i> *	195	104
DD46†	20.25	26.6
DD46 <i>oxrA</i> *	232.9	215
JJ3†	25.1	24.7
JJ3 <i>oxrA</i> *	237.4	181.4
NN19†	36.1	25.2
NN19 <i>oxrA</i> *	195.1	144.7

membrane fraction of anaerobically grown cells (Fig. 2, lanes I, J, L, M, O and P).

As shown in Fig. 2, another chlorate-resistant mutant (MM46) isolated by us also produced the 134.8 and 118.5 kDa proteins when grown under anaerobic conditions (Fig. 2, lanes F and G). This mutant was not characterized further in this work because, in contrast to mutants DD46, NN19 and JJ3, it had a rough lipopolysaccharide (Contreras *et al.*, 1995a), a fact that does not allow the use of phage P22 for transduction experiments.

Growth in semi-solid MIO medium and observation by light microscopy showed that under aerobic conditions the three mutants were motile. However, in contrast to the parental strain, all three mutants were non-motile

Table 4. Effect of *oxrA* on the expression of anaerobically induced operon fusions

Values are expressed as β -galactosidase activity in Miller units (Miller, 1972). Data are from one representative experiment. Each experiment was repeated at least three times.

Strain	+O ₂	–O ₂	–O ₂ /+O ₂	–O ₂ +NO ₃ [–] *	(–O ₂ +NO ₃ [–])/ –O ₂ *
Ty2 <i>chlC</i> ::MudA†	440	1258	2.9	1720	1.4
Ty2 <i>chlC</i> ::MudA <i>oxrA2</i> ::Tn10‡	91	495	5.4	458	0.9
DD46	363	1968	5.4	1890	1.0
DD46 <i>oxrA2</i> ::Tn10	531	920	1.7	1021	1.1
JJ3	477	1841	3.9	1827	1.0
JJ3 <i>oxrA2</i> ::Tn10	390	1277	3.4	1196	0.9
NN19	1201	2520	2.1	3433	1.4
NN19 <i>oxrA2</i> ::Tn10	599	1068	1.8	681	0.6

* Nitrate was added at 20 mM final concentration in LB.

† *S. typhi* strain Ty2 *chlC*::MudA was obtained by phage P22HTint transduction from *S. typhimurium* strain TN3038.

‡ *S. typhi* strain Ty2 *chlC*::MudA *oxrA2*::Tn10 was obtained by phage P22HTint transduction from *S. typhimurium* strain MST2970 to *S. typhi* strain Ty2 *chlC*::MudA.

Fig. 2. SDS-PAGE analysis of outer-membrane-proteins of *S. typhi* strain Ty2 and chlorate-resistant mutants MM46, DD46, NN19 and JJ3. Lane A, molecular mass standards; lanes B, E, H, K and N, bacteria grown in LB under aerobic conditions; lanes C, F, I, L and O, bacteria grown in LB under anaerobic conditions; lanes D, G, J, M and P, bacteria grown under anaerobic conditions in LB supplemented with NaNO_3 .

when grown in nitrate-supplemented LB under anaerobic conditions. Mutant NN19 was unique in that this defect was suppressed by the addition of sodium molybdate to the culture medium. By transmission electron microscopy it was observed that the non-motile bacteria were flagellated (data not shown). We believe that this result could be explained by the lack of nitrate reductase activity as this respiratory enzyme participates in the generation of proton-motive force that drives flagellar movement (Morpeth & Boxer, 1985; Gennis & Stewart, 1996).

DISCUSSION

As part of our interest in the identification of genes that allow *S. typhi* to survive and multiply within the human host, we have isolated a collection of anaerobically induced *lacZ* operon fusions and characterized them with respect to their abilities to enter into and proliferate within HEp-2 epithelial cells (Contreras *et al.*, 1995a). In the present work we have further characterized three of these anaerobically induced operon fusions whose ability to proliferate within HEp-2 cells is considerably diminished compared to the parental strain. All three mutants were unable to grow anaerobically in media containing a non-fermentable substrate (glycerol) with nitrate as the terminal electron acceptor, suggesting that they lack nitrate reductase activity. By phage P22 transduction of a *chlC*::Tn10 insertion from *S. typhimurium* into these mutants, we demonstrated that the MudJ insertions are not located in the *chlC* (*nar*) locus.

The three mutant strains showed a chlorate-resistant phenotype. Other studies have shown that virtually all chlorate-resistant mutants of *E. coli* have alterations in the synthesis or assembly of molybdenum cofactor. Five *mol* (formerly *chl*) loci have been identified; the *moaA*

(*chlA*) and *moe* (*chlE*) gene products are involved in synthesis of molybdopterin (Pitterle & Rajagopalan, 1989), the *mod* (*chlD*) gene product is involved in the active transport of molybdenum (Johann & Hinton, 1987; Miller *et al.*, 1987; Scott & Amy, 1989), the *mob* (*chlB*) gene product is required for molybdenum processing (Johnson *et al.*, 1991) and the *mog* (*chlG*) gene product has an unknown function (Garzón *et al.*, 1992; Rajagopalan & Johnson, 1992). Growth of fusion strain NN19 was restored to wild-type levels in medium supplemented with molybdate, indicating that this is probably a *mod* mutant. The genetic mapping results support this conclusion, namely, the MudJ insertion in mutant NN19 maps in the 17–19 min region of the chromosome and the *S. typhimurium chlD* (*modC*) gene has been mapped at the 18.3 min (Sanderson *et al.*, 1995).

Characterization of *oxrA* derivatives of these mutants indicates that anaerobic induction of fusions NN19 and JJ3 is independent of the *oxrA* gene, suggesting alternative regulation of these loci. In *S. typhimurium*, in addition to the *oxrA* gene (similar to the *fur* gene of *E. coli*) and the *oxrB* gene, which are essential for the anaerobic induction of several respiratory enzymes, two other regulatory genes, *oxrC* and *tppR*, have been described (Jamieson & Higgins, 1986). The *oxrA* mutation, on the other hand, reduced the anaerobic induction of mutant DD46 fusion expression. This result, together with the observation that this mutant is unable to utilize both nitrate and fumarate as terminal electron acceptors anaerobically, suggests that the MudJ insertion is probably affecting a gene which is regulated by the *oxrA* gene and is involved in the anaerobic induction of both nitrate and fumarate reductases in *S. typhi*.

Examination of membrane proteins by SDS-PAGE

showed that the addition of nitrate to wild-type *S. typhi* growing anaerobically in complex medium caused increased synthesis of a protein of approximately 140 kDa. This protein was present in membrane fractions of all the mutants in the absence of nitrate, with the exception of mutant NN19. It is tempting to speculate that this protein might correspond to the α -subunit of nitrate reductase, which in *E. coli* has a molecular mass of 142 kDa (Stewart & MacGregor, 1982). The fact that mutant NN19 does not synthesize this protein supports this notion. In *chlD* mutants of *E. coli*, the band corresponding to nitrate reductase is not found in SDS-PAGE gels of membrane fractions (MacGregor & Schnaitman, 1971). Mutant strain JJ3 might be a *chlA*-type mutant in which the nitrate reductase is present in the membrane fraction but does not function (MacGregor & Schnaitman, 1971). The approximate map position within the 17–19 min region, as well as its nitrate-independent expression, is in accordance with properties reported in *chlA* mutants of other enterobacteria: *S. typhimurium chlA (moaA)* gene maps at 19.0 min on the bacterial chromosome (Sanderson *et al.*, 1995) and transposon insertions in the *chlA* gene result in constitutive *nar-lacZ* expression (Pascal *et al.*, 1982). Finally, mutant DD46 might be a *chlE*-type mutant, which in *E. coli* barely grows in a medium containing glycerol and fumarate (Pascal & Chippaux, 1982).

Although oxygen regulation of protein synthesis has been extensively studied in other facultative anaerobes, such as *E. coli* and *S. typhimurium*, very little is known about the effects of anaerobiosis on gene expression in *S. typhi*, due in part to the lack of adequate tools for genetic analysis in this human pathogen. In the present work we have shown that anaerobically induced genes which are involved in nitrate respiration are required for *S. typhi* invasiveness. The role of these genes in the invasion process may be an indirect one, for example, in providing the most energetically favourable metabolic processes for bacterial survival and proliferation. Alternatively, genes involved in anaerobic respiration may be coordinately regulated with genes encoding invasive capacity. Our results support recent findings, using *in vivo* expression technology, which show that mutations in several *S. typhimurium* genes involved in metabolic pathways result in reduced virulence in mice (Mahan *et al.*, 1993; Moors & Portnoy, 1995). This is in agreement with an expansive view of virulence determinants which includes not only those factors that cause disease, but also those functions that contribute to bacterial survival and multiplication in the host (Mekalanos, 1992; Groisman & Saier, 1990).

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