Mutations that affect the regulation of *phs* in *Salmonella typhimurium*

NEZHA ALAMI and PATRICK C. HALLENBECK*

Département de microbiologie et immunologie, Université de Montréal, Montréal, Québec, Canada, H3C 3J7

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The regulation of *phs* [production of hydrogen sulphide (H_2S)] in *Salmonella typhimurium* is complex. Previous studies have shown that expression is dependent upon the presence of reduced sulphur and anaerobiosis and is modulated by carbon source and growth stage. Transposon mutagenesis failed to find any potential *trans*-acting factors effective in the regulation of *phs* in relation to oxygen. Spontaneous mutants capable of expressing *phs-lac* aerobically were isolated and characterized. These mutations are closely linked to *phs* and affect not only oxygen regulation but also the requirement for cyclic AMP and reduced sulphur. Analysis of merodiploid strains indicates that these mutations are *cis*-acting and that *phs* is not subject to autoregulation.

Introduction

The *Enterobacteriaceae* are facultative anaerobes that can obtain energy for growth through a number of different pathways which are regulated so that, under a given set of growth conditions, the pathway yielding the greatest amount of energy is utilized (Stewart, 1988). Several factors have been shown to be involved in regulation of the cellular response to anaerobiosis. DNA topography appears to play an important role. DNA topoisomerase I (*topI*) mutants cannot grow in the presence of oxygen whereas DNA gyrase (*gyrA*) mutants cannot grow in its absence (Yamamoto & Droffner, 1985). DNA supercoiling *in vivo* is increased by anaerobic growth (Dorman *et al.*, 1988). In addition, the expression of several identified genes, including *tonB* (Dorman *et al.*, 1988), *nifLA* (Dimri & Das, 1988; Dixon *et al.*, 1988), *nifH* (Kranz & Haselkorn, 1986) and *proU*, *ompC* and *tppB* (Ni Bhriain *et al.*, 1989), is increased when gyrase is either chemically inhibited or mutationally inactivated. In contrast, the anaerobic induction of *fdhF* in *Escherichia coli* is enhanced by gyrase inactivation (Axley & Stadtman, 1988).

While changes in DNA topology may serve as a global regulatory signal, regulation of the expression of at least some pathways requires specific *trans*-acting factors. Anaerobiosis represses the synthesis of aerobic pathways via the *arc* system (Iuchi & Lin, 1991). The expression of anaerobic respiratory pathways, including nitrate reductase, requires active Fnr (Oxra in *Salmonella typhimurium*) (Spiro & Guest, 1990). The anaerobic induction

of at least some fermentative enzymes requires other unique factors (Schlensog & Böck, 1990; Fasciano & Hallenbeck, 1991; Rossman *et al.*, 1991); in this case, transcription requires, in addition, RNA polymerase containing NtrA (Birkmann *et al.*, 1987; Fasciano & Hallenbeck, 1991).

Many aspects of the anaerobic metabolism of *S. typhimurium* closely resemble those of *E. coli*. However, unlike *E. coli*, under anaerobic conditions *S. typhimurium* produces H_2S from either thiosulphate [via a thiosulphate reductase (Barrett & Clark, 1987; Clark & Barrett, 1987a)] or from sulphite [via an anaerobically induced sulphite reductase (Hallenbeck *et al.*, 1989)]. Previous studies have defined a genetic locus, *phs*, that appears to be essential for anaerobic thiosulphate reduction *in vivo* and for *in vitro* thiosulphate reductase activity (Clark & Barrett, 1987a, b; Voll *et al.*, 1974, 1979). Temperature-sensitive *phs* mutants do not produce thermolabile thiosulphate reductase, as evidenced by *in vitro* thiosulphate-dependent methyl viologen reduction (Clark & Barrett, 1987a). This suggests that *phs* does not encode the structural gene(s) for thiosulphate reductase but rather an auxiliary function such as synthesis of a required cofactor or the positive control of synthesis of thiosulphate reductase.

Previous studies (Clark & Barrett, 1987a), using *phs* Mud1 operon fusion mutants, have shown that regulation of *phs* expression is complex. Expression of *phs* was reported to be dependent on anaerobiosis and the presence of reduced sulphur. Carbon source and growth stage also affect *phs* expression. The basis of the anaerobic regulation of *phs* is unclear. Some anaerobic respiratory pathways, including nitrate reductase,

* Author for correspondence. Tel. (514) 343 6278; fax (514) 343 5701.

appear to be regulated by *oxrA* (Jamieson & Higgins, 1986; Strauch *et al.*, 1985). However, even though thiosulphate reductase activity is substantially decreased in an *oxrA*⁻ strain, *phs* expression is unaffected by mutations in *oxrA* (Clark & Barrett, 1987a). Catabolite repression of thiosulphate reduction in *S. typhimurium* is complex. A *crp*⁻ mutant did not produce H₂S from thiosulphate and cyclic AMP was essential both for H₂S production from thiosulphate and methyl-viologen-linked thiosulphate reductase activity. However, addition of cyclic AMP did not increase *phs* expression in the presence of some of the sugars tested (Clark & Barrett, 1987b). Anaerobic induction of *phs* (Aliabadi *et al.*, 1986; Clark & Barrett, 1987a) requires complex media which can be partially replaced by the inclusion of various forms of reduced sulphur in the medium (Clark & Barrett, 1987a). Clearly, the regulation of *phs* expression is complex and requires further studies. Here we report on mutants, derived from *phs-lac* operon fusions, that are capable of aerobic *lac* expression. We have also investigated the regulation of wild-type *phs-lac* operon fusions and the mutants described in the present study in merodiploid strains.

Methods

Strains, media and growth conditions. The strains of *S. typhimurium* used in this study are listed in Table 1. Rich media, LB or NB, were as described by Miller (1972). The minimal medium was M9 minimal base (Davis *et al.*, 1980) with 0.1% galactose. Lactose minimal medium for selection of lactose utilization was M9 base with 0.5% lactose. MacConkey agar, nutrient broth and peptone iron agar were from Difco. The ability to produce H₂S was tested on peptone iron agar with an overlay of the same medium. Antibiotics were used at the following final concentrations: ampicillin, 50 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; tetracycline, 25 µg ml⁻¹. Minimal media were formulated with half these concentrations. Anaerobic growth was achieved by filling tubes to the top and stoppering after inoculation and aerobic growth was either by incubating partially filled 16 × 25 mm tubes on a roller drum, or by shaking (300 r.p.m.) loosely capped tubes. All cultures were grown at 37 °C.

Genetic methods. Genetic manipulations followed standard procedures (Davis *et al.*, 1980) and transductions were performed with P22 *int4* (Smith & Levine, 1967).

We screened for insertion mutants using pools of random Tn10 and Tn5 insertions kindly provided by J. R. Roth (Dept of Biology, Univ. of Utah, USA) and S. Artz (Dept of Bacteriology, Univ. of California, Davis, USA), respectively. The random Tn10 insertion clones were obtained by infecting LT2 with a P22 phage that was defective for replication and carried Tn10 in its genome. Tetracycline-resistant colonies (5000), each of which is the result of an independent insertion, were pooled (Davis *et al.*, 1980). The random Tn5 insertion clones were obtained by making a phage lysate of strain TT3416 [*pyrB655/Fts 114lac*(+) *zzf-771::Tn5*]. Subsequent transduction of LT2 and selection for kanamycin resistance yields clones that have inherited Tn5 by transposition. Approximately 5000 clones were pooled. To screen for a given insertion mutant, phage lysates of the pooled clones are prepared (Davis *et al.*, 1980). The appropriate strain is transduced

to either tetracycline (Tn10) or kanamycin (Tn5) resistance and screened for the desired phenotype. Here, strain EB222 (*phs101::Mud1*) was the recipient strain, and transductants were screened for a change in colony colour on MacConkey lactose (plus antibiotic) plates.

Spontaneous mutants of strain EB222 capable of aerobic β-galactosidase expression were selected by plating appropriate volumes of overnight cultures on aerobically incubated lactose minimal plates. Putative mutants were purified on the same medium and checked for red colony colour on MacConkey lactose plates. Strains diploid for the *phs* locus were created by transducing the mutant *phs* alleles from strains EB331–335 into strain TT13274 and selecting for ampicillin resistance. This strain is diploid for the entire region between *cob* and *his* and thus *phs*. We verified that the resultant diploid strains contained the mutant alleles by marker rescue.

Biochemical methods. β-Galactosidase activities of mid-exponential phase cultures were assayed by a modification (Putnam & Koch, 1975) of the method of Miller (1972) and expressed as nmol *o*-nitrophenol produced min⁻¹ (OD₆₀₀ unit)⁻¹ at 28 °C. All assays were done in duplicate and reported values are means from at least three independent experiments.

Results

Isolation and characterization of phs regulatory mutants. Insertion mutants

We screened, on MacConkey plates containing either tetracycline or kanamycin, pools of random Tn10 and Tn5 insertions for possible regulatory mutations leading to a change in *phs* expression. If the normal lack of expression under aerobic conditions was due to a repressor, we expected to find increased expression on aerobically incubated MacConkey plates. Conversely, we screened for the inactivation of anaerobic activators of *phs* by looking for decreased colony colour on anaerobically incubated plates. In both cases, no such mutants were detected. However, several Tn10 insertions (PH54–56) were found that increased anaerobic *phs* expression 1.6–1.8-fold in minimal or rich medium without affecting the decrease in *phs* expression by glucose. These insertions were relatively specific for *phs* since they had no effect on the anaerobic expression of *hyd*, *fdhF* or *pepT* fusions. These Tn10 insertions are unlinked to *phs* (results not shown).

Isolation and characterization of spontaneous mutants capable of aerobic phs expression

Little β-galactosidase is expressed by an aerobically grown *phs::Mud1* mutant, strain EB222 (Clark & Barrett, 1987a) and this strain is incapable of aerobic growth on lactose as the sole source of carbon. Spontaneous mutants capable of aerobic *lac* expression (lactose utilization) were selected. They arose at a frequency of 6.5 × 10⁻⁸. Five spontaneous mutants

Table 1. *Strains of S. typhimurium used in this work*

Strain	Relevant genotype or phenotype†	Source or reference‡
EB219	<i>cya-1091::Tn10</i>	Clark & Barrett (1987b)
EB222	<i>phs-101::Mud1</i>	Clark & Barrett (1987a)
EB243	<i>cya-1091::Tn10 phs-101::Mud1(Ap^rlac)</i>	Clark & Barrett (1987b)
EB331–335	<i>phs-201::Mud1 to phs-205::Mud1</i> [spontaneous Lac ⁺ (aerobic) mutants of EB222]	This study
EB337–341	As for EB331–335, but containing <i>cya-1091::Tn10</i>	P22 (EB219) × EB331–335
PH54	<i>phs-101::Mud1(Ap)</i> <i>zxx-5101::Tn10</i>	P22 (RnTn10) × EB222
PH56	<i>phs-101::Mud1(Ap)</i> <i>zxx-5102::Tn10</i>	P22 (RnTn10) × EB222
PH308–312	<i>phs-206::Mud1 to phs-210::Mud1</i> [spontaneous Lac ⁺ (aerobic) mutants of EB222]	This study
TT13274	<i>metE205 ara-9</i> Dup [(<i>hisF9951</i>)*Tn10*(<i>cob-20</i>)]	J. R. Roth, Univ. of Utah, USA

† Insertions of transposable elements into known genes are indicated by the gene and allele number, followed by :: and the insertion element. When the location of the insertion is not known this is indicated by *zxx*. Dup indicates a duplication with the extent of the duplication indicated by the genes in parentheses.

‡ When strains are transductants, this is indicated by P22 (*y*) × *z* where *y* is the strain used to prepare the phage lysate and *z* is the recipient strain. RnTn10 indicates a random pool of Tn10 insertions.

Table 2. *Effect of growth conditions on β-galactosidase expression of mutant strains that express phs::Mud1 aerobically*

NB, nutrient broth; NB-Glucose, nutrient broth with 10 mM-D-glucose; M9, M9 minimal medium.

		β-Galactosidase activity [nmol o-nitrophenol min ⁻¹ (OD ₆₀₀ unit) ⁻¹]			
Mutant selection conditions	Strain	Aerobic growth		Anaerobic growth	
		NB	NB	NB-Glucose	M9
Aerobic, lactose	EB222*	14	260	39	6
	EB331	81	67	144	126
	EB332	939	784	724	440
	EB333	170	489	216	329
	EB334	208	444	222	429
Aerobic, lactose, 1 mM-S ²⁻	EB335	240	369	291	604
	PH308	209	248	379	101
	PH309	156	172	138	96
	PH310	182	182	123	106
	PH311	197	165	137	133
	PH312	232	216	121	142

* Strain EB222 is the *phs-lac* parent from which all other strains were selected on the basis of the aerobic expression of *lac*.

the transductants were scored, on MacConkey lactose media, for co-inheritance of aerobic expression of *phs*. All ampicillin-resistant transductants had also inherited the H₂S⁻ phenotype indicating that the aerobic phenotype was not due to transposition of the immobilized Mud1 to a different location. In all five, the mutation allowing aerobic expression was greater than 68% (68–99% depending upon the strain) linked to ampicillin resistance. This linkage is very high considering that P22 can only transfer about 44 kb of DNA, and, in this case, the fusion itself constitutes 36 kb. In other words, when considering the frequency of co-inheritance of the mutation and the fusion, one must take into account the quantity of non-homologous DNA that interrupts the homologous DNA that must recombine to have a successful transduction. Applying the appropriate formula developed for this circumstance (Sanderson & Roth, 1988), one can calculate that co-transduction frequencies of 68–97% correspond to physical differences of approximately 1000–100 base pairs.

Expression of β-galactosidase by these mutants (strains EB331–335) was determined under a variety of growth conditions (Table 2). All mutants produced more β-galactosidase than the parent strain (EB222) under aerobic growth conditions, but the level of expression varied from 6-fold (EB331) to 60-fold (EB332) greater than EB222. Additionally, the large anaerobic induction (19-fold) seen with EB222 was no longer apparent. As noted above, catabolite regulation of thiosulphate reduction is complex. Mutant strains EB331–335 appeared to

(EB331–335) derived from EB222 were further characterized. For each mutant, the P22 co-transductional linkage of the mutation allowing aerobic expression to the *phs-lac* fusion was determined. Resistance to ampicillin was transferred to LT2 by transduction, and

Table 3. Effects of glucose and cyclic AMP on *phs-lac* expression

NB, nutrient broth; NB-Glucose, nutrient broth with 10 mM-D-glucose; %NB, percentage activity obtained without glucose.

Cya ⁻ strain	(Cya ⁺ parent)	β -Galactosidase activity [nmol <i>o</i> -nitrophenol min ⁻¹ (mg protein) ⁻¹]			
		No cyclic AMP		+ 10 mM cyclic AMP	
		NB	NB-Glucose (%NB)	NB	NB-Glucose (%NB)
EB243	(EB222)	29	33 (114)	209	71 (34)
EB337	(EB331)	300	189 (63)	229	110 (48)
EB338	(EB332)	918	833 (91)	1231	1033 (84)
EB339	(EB333)	547	291 (53)	722	313 (43)
EB340	(EB334)	516	396 (77)	562	476 (84)
EB341	(EB335)	918	604 (66)	680	404 (59)

have lost some or all of the effect of glucose on anaerobic expression of *phs*. This is treated in more detail in the following section. Finally, these mutants had also lost to a great extent the requirement for reduced sulphur for anaerobic induction in minimal media, a requirement that is characteristic of the parent strain EB222 (Table 2). To rule out that this was due to the selection procedure used, we carried out a second round of selection in which sulphide (1 mM) was added to the minimal lactose medium. However, this selection yielded mutants (PH308–312) with generally similar regulatory alterations (Table 2) to those of the mutant strains previously described (EB331–335). P22 transductional analysis showed that the mutations in strains PH308–312 were, like those of EB331–335, highly linked to the *phs-lac* fusion.

Effect of cyclic AMP on glucose repression

To examine more closely the effects of glucose and cyclic AMP on *phs-lac* expression, a *cya::Tn10* mutation was introduced into each of the isolated strains and the resulting double mutants assayed for β -galactosidase activity with or without cyclic AMP and glucose (Table 3). Expression of *phs* by the parent operon fusion was greatly reduced by the presence of a *cya* mutation (compare EB243 with EB222 in the absence of cyclic AMP) and glucose was repressive even in the presence of 10 mM cyclic AMP. In contrast, all of the aerobic expression mutants were unaffected by the *cya* mutation (Table 3). Addition of 10 mM cyclic AMP resulted in an 8-fold increase in expression by EB243, but had little or no effect on EB337–341. In these strains, glucose reduced *phs* expression in the presence or absence of cyclic AMP (Table 3) to an approximately similar value to that observed in a *cya*⁺ background (Table 2).

Regulation of *phs* in diploid strains

The mutations that permitted aerobic expression of *phs-lac* proved to be highly linked to the fusion itself. To determine whether the mutations were *cis*- or *trans*-acting, strains that were diploid for the *phs* locus, i.e. *phs-101/phs*⁺, *phs-201/phs*⁺ etc., were constructed by transduction of the required alleles into TT13274 (obtained from J. R. Roth), which carries a duplication of the chromosomal region between 41 (*cob*) and 42 (*his*) min. Diploid strains were simultaneously *Phs*⁺ (wild-type gene) and *Amp*^R (*phs-lac* fusion). Inheritance of the altered *phs* allele was verified by marker rescue. The β -galactosidase activities of these strains, grown under various conditions (nutrient broth and oxygen, nutrient broth minus oxygen; with and without glucose) were compared with those of the respective haploid strains (results not shown). No differences were observed, including those for the parent strain (EB222; *phs-101::Mud1*). This strongly suggests: (1) that all the selected mutations were *cis*-acting, i.e. probably in or near the *phs* promoter operator region; and (2) that the gene in which the original insertion was isolated is not autoregulated. Since the *phs* locus appears to play a part in the regulation of thiosulphate reductase, we assayed thiosulphate reductase activity, using a previously described method (Hallenbeck *et al.*, 1989), and again found no difference between the diploid strains described above and the *phs*-haploid strain EB222 (results not shown).

Discussion

The pathway of anaerobic reduction of thiosulphate to H₂S and the associated regulation of *phs* are of interest because of the significant differences in regulation of thiosulphate reduction and other anaerobic systems, such as catabolite repression of *phs* and its lack of response to an *oxrA* mutation (Clark & Barrett, 1987*a, b*) or a *ntrA* mutation (Fasciano & Hallenbeck, 1991). Previous studies have shown that regulation in *S. typhimurium* of thiosulphate reduction is complex (Clark & Barrett, 1987*a*). DNA supercoiling does not, however, seem to play a role since inhibition of DNA gyrase with novobiocin (50 μ g ml⁻¹) has no effect on *phs* expression (unpublished results). The structural gene(s) for thiosulphate reductase have not, as yet, been identified. The genetic locus *phs* is absolutely required for production of active thiosulphate reductase activity. One hypothesis is that *phs* encodes a regulatory protein required for anaerobic expression of thiosulphate reductase (Clark & Barrett, 1987*a*). The regulation of *phs* itself appears to be complex (Clark & Barrett, 1987*a, b*) and the genetic factors involved have yet to be identified.

In the present study we attempted, using transposon mutagenesis, to find *trans*-acting factors responsible for the regulation of *phs*. We expected to find either increased aerobic expression due to inactivation of a repressor or decreased anaerobic expression due to inactivation of an activator. Neither type of mutant was found. Several isolates showed modestly increased anaerobic expression of *phs* but it seems likely that the defect has an indirect effect on *phs* expression because the overall regulatory pattern remains the same as in the absence of the insertion. Spontaneous mutations causing aerobic expression of *phs* all mapped in the *phs* region and were highly linked to the previously described *phs-lac* fusion. These mutations affect not only oxygen regulation but also the requirement by *phs* for cyclic AMP and complex media. Therefore these mutations may lie in a *phs* promoter/operator region constituting a locus where all three signals interact. Among other possibilities, these mutations could be due to single base changes, deletions that bring the *phs* gene under the control of a constitutive promoter upstream of the *phs* gene or deletions of the *phs* promoter region that abolish repressor binding. Studies are currently in progress to determine their physical nature. Nevertheless, the spontaneous mutants were freed simultaneously from the three controls of *phs* expression. This indicates that regulation of thiosulphate reductase by these parameters may be at least partially explained by their effects on the synthesis of a *phs*-encoded protein which, in turn, induces the thiosulphate reduction pathway. In contrast, synthesis of the Fnr protein, which is needed to induce several anaerobic electron transport systems (Jamieson & Higgins, 1986; Jones & Gunsalus, 1985; Strauch *et al.*, 1985) is not itself regulated by aerobiosis (Pascal *et al.*, 1986). When the structural genes for components of the thiosulphate reduction pathway have been identified, it will be interesting to see if its *phs*-mediated regulation can be separated from regulation by *oxrA* and nitrate, both of which have a much greater effect on H₂S production than on thiosulphate reductase synthesis.

Glucose and other sugars decrease β -galactosidase expression by strain EB222 (Clark & Barrett, 1987*a, b*). The characteristics of the aerobic expression mutants isolated here support the hypothesis (Clark & Barrett, 1987*b*) that catabolite repression of thiosulphate reduction involves two modes of regulation. Expression of thiosulphate reductase in a *phs*⁺ strain and of β -galactosidase in the *phs-lac* operon fusion parent showed an absolute requirement for *cya* gene function. This requirement was abolished in the aerobic expression mutants isolated here, but β -galactoside expression was still repressed by glucose even in the presence of the *cya* mutation (Table 3). The retention of glucose repression in the absence of a need for *cya* constitutes strong

evidence for two separate mechanisms of glucose repression of *phs* expression: a pronounced repression attributable to cyclic-AMP-mediated catabolite repression (abolished in the mutants capable of aerobic *phs* expression), and a moderate repression mediated by another mechanism (retained in these mutants). The negative regulation of thiosulphate reduction by carbon sources from which highly reduced electron donors such as formate and hydrogen are derived (Clark & Barrett, 1987*b*) is somewhat of a paradox in light of the fact that the redox potential for the thiosulphate/sulphite-sulphide couple is too low for energetic electron transport from most other electron carriers such as glycerol phosphate and NADH (Barrett & Clark, 1987).

Finally, one possibility that had not yet been examined was that *phs* was subject to autoregulation. To test this possibility, we constructed, using P22 transduction and a strain diploid for the *phs* region, strains that were *phs*⁺ and carried various *phs-lac* fusion alleles. However, we observed no effect of diploidy on β -galactosidase expression under a variety of conditions: thus all the mutations were *cis*-acting. Among several possibilities, the results presented here suggest that either: (1) a *trans*-acting factor exists but was not detected due to the selection procedures used, or (2) that *phs* is subject to a novel type of *cis*-acting regulation. Studies are presently in progress to address these questions on a molecular level.

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