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Effects of Anaerobic Regulatory Mutations and Catabolite Repression on Regulation of Hydrogen Metabolism and Hydrogenase Isoenzyme Composition in *Salmonella typhimurium*

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Hydrogen metabolism in *Salmonella typhimurium* is differentially regulated by mutations in the two anaerobic regulatory pathways, defined by the *fnr* (*oxrA*) and *oxrC* genes, and is controlled by catabolite repression. The synthesis of the individual hydrogenase isoenzymes is also specifically influenced by *fnr* and *oxrC* mutations and by catabolite repression in a manner entirely consistent with the proposed role for each isoenzyme in hydrogen metabolism. Synthesis of hydrogenase isoenzyme 2 was found to be *fnr* dependent and *oxrC* independent, consistent with a role in respiration-linked hydrogen uptake which was shown to be similarly regulated. Also in keeping with such a respiratory role was the finding that both hydrogen uptake and the expression of isoenzyme 2 are under catabolite repression. In contrast, formate hydrogenlyase-dependent hydrogen evolution, characteristic of fermentative growth, was reduced in *oxrC* strains but not in *fnr* strains. Hydrogenase 3 activity was similarly regulated, consistent with a role in hydrogen evolution. Unlike the expression of hydrogenases 2 and 3, hydrogenase 1 expression was both *fnr* and *oxrC* dependent. Hydrogen uptake during fermentative growth was also both *fnr* and *oxrC* dependent. This provided good evidence for a distinction between hydrogen uptake during fermentation- and respiration-dependent growth and for a hydrogen-recycling process. The pattern of anaerobic control of hydrogenase activities illustrated the functional diversity of the isoenzymes and, in addition, the physiological distinction between the two anaerobic regulatory pathways, anaerobic respiratory genes being *fnr* dependent and enzymes required during fermentative growth being *oxrC* dependent.

Escherichia coli and *Salmonella typhimurium* each possess at least three distinct hydrogenase isoenzymes (22, 24), which together are responsible for both hydrogen uptake and hydrogen-evolving activities. The hydrogen uptake (respiratory) activity is involved in anaerobic energy generation (1, 8), whereas the hydrogen-evolving reaction is catalyzed by the formate hydrogenlyase system (20). Two membrane-bound hydrogenases, isoenzymes 1 and 2, have been purified from *E. coli* and characterized as nickel-containing metalloenzymes (2, 3, 23). *S. typhimurium* also contains functional equivalents to isoenzymes 1 and 2 which are immunologically related to their *E. coli* counterparts (24). A third hydrogenase activity, immunologically distinct from isoenzymes 1 and 2, is also present in membranes from anaerobically grown *E. coli* and *S. typhimurium* (23, 24). Previously (24), we identified distinct physiological roles for these three isoenzymes. Isoenzymes 1 and 2 catalyze hydrogen uptake reactions; isoenzyme 1 functions during fermentative growth, whereas isoenzyme 2 is active only during respiration-dependent growth. Hydrogenase 3 catalyzes hydrogen evolution and appears to form part of the formate hydrogenlyase pathway.

Hydrogen is metabolized by enterobacteria only during anaerobic growth. We have identified two genetically and physiologically distinct regulatory pathways for the anaerobic control of gene expression (7). The *fnr* (25) (designated *oxrA* in *S. typhimurium* [28]) and *oxrC* genes are two pleiotropic regulatory loci which define two distinct classes of anaerobically induced genes. We have suggested that *fnr* may control the expression of anaerobic respiratory genes (5, 9, 10, 16, 27), whereas *oxrC* principally regulates the synthesis of enzymes with fermentative or biosynthetic roles (7). Because the hydrogenases serve both respiratory and fermentative functions (24), we examined the effects of *fnr* and *oxrC* mutations on hydrogen metabolism and on the synthesis of the individual hydrogenase isoenzymes. Each of the hydrogenase isoenzymes was shown to be subject to distinct control processes, which provides strong support for the physiological roles proposed for the enzymes (24) and for the distinct physiological roles of the *fnr* and *oxrC* regulatory pathways.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study as well as their genotypes and construction are detailed in Table 1.

Media and growth conditions. Cells were grown anaerobically in nutrient broth (NB; Difco Laboratories) at 37°C unless otherwise stated. Anaerobic growth was without shaking in completely filled screw-cap vessels, or, alternatively, GasPaks (Oxoid Ltd.) were used to provide an anaerobic environment. LB medium was described by Miller (14). NB was supplemented when appropriate with additional carbon sources at 0.4% or with other additives as follows: sodium nitrate, 10 g liter^{-1}; sodium formate, 0.5 g liter^{-1}; and sodium fumarate, 5 g liter^{-1}. Ammonium molybdate and K_2SeO_3 were added to all growth media at 1 μM. Kanamycin and ampicillin were used at 50 μg ml^{-1}. MacConkey agar-nitrate and glycerol-nitrate plates were as described by Barrett et al. (4) and Lambden and Guest (10), respectively.

Genetic techniques. Transductions were performed by

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TABLE 1. Bacterial strains

Strain[a]	Genotype[b]	Source
S. typhimurium		
LT2 (Z)	Wild type	B. N. Ames
CH602	*pepT7*::Mu dl *oxrA1* *zda-893*::Tn*5*	7, 28
CH805	Δ*oppBC250 tppB84*::Mu d1-8 *oxrC102*::Tn*5*	7
CH953	*hyd*::Mu dl(Ap *lac*) *oxrC102*::Tn*5*	7
CH975	*hyd*::Mu dl(Ap *lac*) *oxrA1* *zda-893*::Tn*5*	7
CH1019	*oxrA1 zda-893*::Tn*5*	7
CH1021	*oxrC102*::Tn*5*	7
CH1107	*cya*::Tn*10*	This study
CH1108	*crp*::Tn*10*	This study
E. coli P4X	Hfr *metB1*	E. Wollman

[a] All *S. typhimurium* strains are derivatives of LT2 (Z) except for CH602 and CH805, which are LT2 (A) derivatives.
[b] *oxrA* is equivalent to the *fnr* locus of *E. coli* (7, 28). The Mu dl(Ap *lac*) fusions in CH953 and CH975 are stabilized.

using a high-transducing derivative of P22 *int-4*. *fnr* (*oxrA*) mutations were moved into appropriate recipients by transduction to Kan^r with a P22 lysate of CH602 (*zda*::Tn*5*; 70% linked to *fnr* [*oxrA*][7, 28]). Cotransfer of the *fnr* allele with the Tn*5* was determined by screening Kan^r transductants for the inability to grow anaerobically on glycerol-nitrate plates and for their growth as small, deep red colonies on anaerobic MacConkey agar-nitrate plates. The *oxrC* mutation was moved between strains by transduction to Kan^r with a P22 lysate of CH805 (*oxrC102*::Tn*5*). Transductants were shown to have acquired the OxrC phenotype by screening for white colonies on MacConkey agar-glucose plates (7). The *cya*::Tn*10* and *crp*::Tn*10* mutations were transferred from strains PP1002 and PP1037 (P. W. Postma, BCP Institute, Amsterdam), respectively, by transduction to Tet^r with P22 lysates of these strains. The acquisition of *cya* and *crp* was confirmed by the loss of the ability to grow anaerobically on minimal medium-glycerol plates. After any transductions involving transposable elements, the correct location of the transposon was checked genetically by marker rescue.

Enzyme assays. All enzyme assays were performed as described previously (24). Hydrogenase activity was measured as the hydrogen-dependent reduction of benzyl viologen. A unit of activity is 1 μmol of benzyl viologen reduced per min. Hydrogen uptake was measured as fumarate-dependent hydrogen uptake. A unit represents 1 μmol of hydrogen oxidized per min. Formate hydrogenlyase activity was monitored as the formate-dependent evolution of hydrogen. A unit represents 1 μmol of hydrogen evolved per min. A unit of formate dehydrogenase (FDH-BV) activity is 1 μg ion of formate oxidized per min (24). A unit of fumarate reductase activity represents 1 μmol of fumarate reduced per min (24). Nitrate reductase was assayed as the nitrate-dependent oxidation of reduced benzyl viologen (15); a unit of activity is 1 μg ion of nitrate reduced per min. β-Galactosidase was assayed as described by Miller (14). Protein was estimated by the method of Lowry et al. (12).

Immunological techniques. Antibodies to *E. coli* hydrogenase isoenzymes 1 and 2 were raised in rabbits, and the immunoglobulin fractions were purified as described previously (2, 23). Rocket immunoelectrophoresis was performed as described previously (6). Immunological quantitation of the hydrogenase activity in Triton X-100-dispersed membranes, which is associated with either isoenzyme 1 or isoenzyme 2, was as described previously (24). The remaining activity, not precipitable with antibodies against either isoenzyme 1 or 2, is termed hydrogenase 3 and was also estimated as described previously (24). Activities of the individual hydrogenase isoenzymes in Triton X-100-dispersed membranes are expressed as micromoles of benzyl viologen reduced per minute per milligram of protein.

RESULTS

Because LT2 (Z) is the only wild-type isolate of *S. typhimurium* which has all three hydrogenase isoenzymes (24), all studies were performed with isogenic derivatives of this strain unless otherwise indicated. Mutations in *oxrC* and *fnr* (*oxrA*) were transduced into LT2 (Z) as described in Materials and Methods. When total hydrogenase activity (H_2:benzyl viologen oxidoreductase) was examined, neither *fnr* nor *oxrC* mutations had major effects (Table 2). However, when hydrogenase activity was separated into its component parts, specific effects of these mutations could be demonstrated.

Regulation of formate hydrogenlyase activity. Formate hydrogenlyase activity (hydrogen evolution) was present in cells grown fermentatively (glucose) and was further induced by added formate (Table 2). However, activity was essentially absent during respiration-dependent growth (glycerol-formate medium). The *oxrC* and *fnr* mutations had very different effects on formate hydrogenlyase activity. Mutations in *oxrC* resulted in a large reduction in activity and, although activity could be restored to some extent by the addition of exogenous formate, it never reached wild-type levels. This implies that formate hydrogenlyase is induced both by anaerobiosis and by formate; the *oxrC* mutation prevents anaerobic induction but has little effect on induction by exogenous formate. Expression of the *fhl* gene is similarly regulated (7). Unlike *oxrC* mutations, mutations in *fnr* only caused a slight reduction in formate hydrogenlyase activity which was restored to normal levels by formate. We have reported a similar, although somewhat more marked, effect in *E. coli* (22). It seems likely that *fnr* does not affect

TABLE 2. Effect of mutations in the anaerobic regulatory genes *oxrC* and *fnr* (*oxrA*) on hydrogen metabolism

Growth conditions and strains[a]	Sp act (U/mg of protein) of[b]:		
	Hydrogenase	Formate hydrogenlyase	Hydrogen uptake
Glucose			
LT2 (Z)	0.449	0.412	0.146
CH1019 (*fnr*)	0.103	0.320	<0.001
CH1021 (*oxrC*)	0.129	0.054	<0.001
Glucose + formate			
LT2 (Z)	0.475	0.699	0.091
CH1019 (*fnr*)	0.260	0.592	<0.001
CH1021 (*oxrC*)	0.711	0.277	<0.001
Glycerol + fumarate			
LT2 (Z)	0.164	0.022	0.377
CH1019 (*fnr*)	0.049	0.059	0.039
CH1021 (*oxrC*)	0.327	<0.001	0.367

[a] Cells were harvested at the late exponential phase of growth, washed once, resuspended in 100 mM potassium phosphate (pH 6.8), and immediately assayed for the activities listed above.
[b] Shown are specific activities for whole cells. For definitions of units of activity, see Materials and Methods.

the anaerobic induction of formate hydrogenlyase directly but that the effect is an indirect consequence of a reduction of endogenous formate synthesis, presumably brought about by a reduction of pyruvate formate lyase activity (22). Thus, the anaerobic induction of formate hydrogenlyase activity is *oxrC* dependent and *fnr* independent.

Regulation of hydrogen uptake. Previously (24), we presented evidence which suggests that *E. coli* and *S. typhimurium* possess two distinct hydrogen uptake activities catalyzed by different isoenzymes, one functioning during fermentative growth and the other functioning during respiration-dependent growth. An examination of the effects of *oxrC* and *fnr* mutations on hydrogen uptake strongly supported this conclusion. Mutations in *oxrC* had no effect on hydrogen uptake during respiration-dependent growth (glycerol-fumarate medium; Table 2). However, hydrogen uptake in cells grown fermentatively, with or without formate, was completely abolished. This is in contrast to the effects of *fnr* mutations, which abolish hydrogen uptake under all growth conditions. Thus, the *oxrC* mutation provides a clear distinction between the two modes of hydrogen uptake.

Effects of *oxrC* and *fnr* mutations on hydrogenase isoenzyme content. There are at least three distinct hydrogenase isoenzymes in *E. coli* and *S. typhimurium* (24). Because of the differential effects of *oxrC* and *fnr* mutations on the different modes of hydrogen metabolism, it was important to assess the effects of these mutations on each individual hydrogenase isoenzyme (Table 3). Mutations in *oxrC* dramatically reduced isoenzyme 1 activity, even in the presence of endogenous formate which, in the wild-type strain, was an inducer of isoenzyme 1 expression. Conversely, *oxrC* mutations had no effect on isoenzyme 2 activity and did not impair the characteristic enhancement of isoenzyme 2 during respiratory growth (glycerol-fumarate medium). The effects of *oxrC* mutations are distinct from those of *fnr* mutations, which reduce both hydrogenase 1 and 2 activities (Table 2).

TABLE 3. Effect of mutations in the anaerobic regulatory genes *oxrC* and *fnr* (*oxrA*) on hydrogenase isoenzyme content

Growth conditions and strains[a]	Hydrogenase sp act (U/mg of protein) of[b]:		
	Isoenzyme 1	Isoenzyme 2	Nonimmunoprecipitable activity
Glucose			
LT2 (Z) (wild type)	0.115	0.655	0.315
CH1019 (*fnr*)	<0.005	0.187	0.255
CH1021 (*oxrC*)	<0.005	0.806	0.272
Glucose + formate			
LT2 (Z) (wild type)	0.097	0.332	1.980
CH1019 (*fnr*)	<0.005	0.077	1.817
CH1021 (*oxrC*)	<0.012	0.726	0.569
Glycerol + fumarate			
LT2 (Z) (wild type)	<0.005	1.25	0.684
CH1019 (*fnr*)	<0.005	0.242	0.827
CH1021 (*oxrC*)	<0.005	1.897	0.452

[a] Cells were harvested, and the membrane fractions were prepared as described in Materials and Methods.

[b] Expressed per milligram of protein in the Triton X-100-dispersed membrane fractions. Isoenzyme 1 and 2 activities were calculated from the activities immunoprecipitated with antibodies specific for isoenzyme 1 or 2, respectively. The nonimmunoprecipitable activity was that activity not immunoprecipitated by a mixture of antibodies specific for isoenzymes 1 and 2.

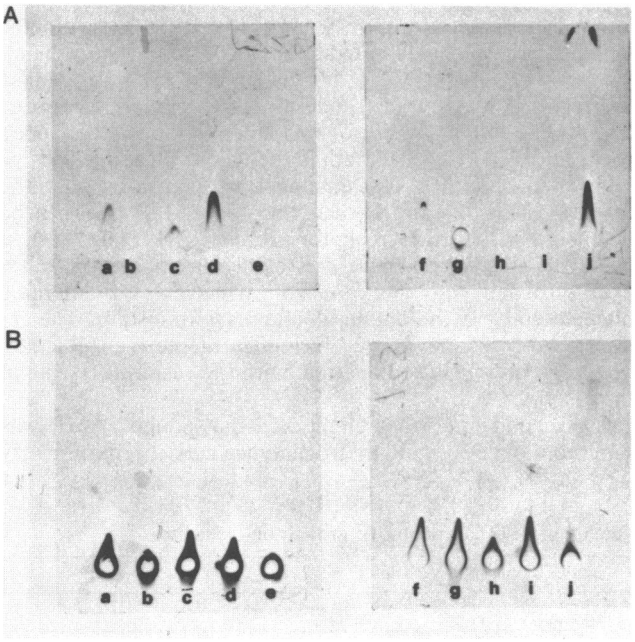

FIG. 1. Hydrogenase isoenzyme 1 and 2 antigen contents of strains carrying lesions in the *oxrC* and *fnr* (*oxrA*) genes. Triton X-100-dispersed membrane fractions (40 μg of protein) were analyzed by rocket immunoelectrophoresis with antibodies to *E. coli* hydrogenase isoenzyme 1 (A) or *E. coli* hydrogenase isoenzyme 2 (B). Lanes: a, LT2 (Z) grown with glucose; b, as for lane a, but CH1019 (*fnr*); c, as for lane a, but CH1021 (*oxrC*); d, LT2 (Z) grown with glucose plus formate; e, as for lane d, but CH1019 (*fnr*); f, as for lane d, but CH1021 (*oxrC*); g, LT2 (Z) grown with glycerol plus fumarate; h, as for lane g, but CH1019 (*fnr*); i, as for lane g, but CH1021 (*oxrC*); j, *E. coli* P4X grown with glucose plus formate.

The presence of hydrogenase 1 and 2 antigens in the *oxrC* and *fnr* mutants was also analyzed immunologically (Fig. 1). In all cases, the presence or absence of antigens corresponded with the differences in isoenzyme activity. This implies that the regulation of hydrogenase isoenzyme composition is at the level of synthesis (transcription/translation) rather than by modulation of enzyme activity.

The hydrogenase activity not associated with isoenzymes 1 and 2 (hydrogenase 3) was somewhat reduced by the *oxrC* mutation, and the formate induction of this activity was also impaired. In contrast, *fnr* mutants had essentially normal and fully formate-inducible hydrogenase 3 activity, although, in the absence of formate, *fnr* mutations did result in a slight reduction in hydrogenase 3 activity. This has also been observed for *E. coli* (22) and is probably an indirect effect caused by reduced synthesis of endogenous formate in the *fnr* mutant. Mutations in the *tppR* gene, which affect a subset of *oxrC*-dependent genes (7), had no effect on cellular hydrogen metabolism or on the synthesis of the individual hydrogenase isoenzymes (data not shown).

The above results, together with the effects of *oxrC* and *fnr* mutations on hydrogen metabolism (see above), are entirely consistent with the proposed role for each of the individual isoenzymes. Hydrogenase 2 is believed to catalyze respiration-dependent hydrogen uptake (24). In keeping with this, *fnr* mutants, but not *oxrC* mutants, were found to have reduced respiration-dependent hydrogen uptake and reduced hydrogenase 2 activity. Similarly, hydrogenase 1 is believed to participate in hydrogen uptake under fermentative growth conditions. The reduction in this activity by *fnr* and *oxrC* mutations can be entirely explained by their similar effects on the cellular content of isoenzyme 1. Finally, the reduction in formate hydrogenlyase activity

TABLE 4. Enzymological characterization of strains carrying mutations in *oxrC* and *fnr*[a]

Strain	Relevant genotype	Sp act (U/mg of protein) of:			
		Nitrate reductase	FDH-BV[b]	Hydrogenase	Fumarate reductase
LT2 (Z)	Wild type	3.24	0.11 (0.16)	3.31	2.35
CH1019	*fnr*	0.04	0.04 (0.17)	1.37	0.14
CH1021	*oxrC*	3.68	0.026 (0.06)	1.17	4.21

[a] Cells were grown anaerobically in NB supplemented with glucose (and KNO_3 when nitrate reductase was assayed), and the membrane fractions were prepared as described in Materials and Methods. All assays were performed on the membrane fraction except for that for FDH-BV for which intact cells were used. For definitions of units of activity, see Materials and Methods.

[b] Values in parentheses refer to FDH-BV activity in whole cells grown in NB supplemented with glucose and sodium formate.

caused by mutations in *oxrC*, but not by mutations in *fnr*, parallels the effects of these enzymes on hydrogenase 3 activity, in agreement with the proposed participation of hydrogenase 3 in hydrogen evolution.

Regulation of formate dehydrogenase activity. Because formate hydrogenlyase requires the function of FDH-BV, as well as a hydrogenase, it was necessary to examine the effects of *oxrC* and *fnr* mutations on this activity. In complete agreement with its minor effect on formate hydrogenlyase activity, an *fnr* mutation only resulted in a small reduction in FDH-BV activity and this could be restored to normal levels by the addition of formate (Table 4). The effects of an *oxrC* mutation were, however, more pronounced. Thus, *oxrC* mutations reduced FDH-BV levels, and activity could not be restored by formate. This is again consistent with the effects of *oxrC* on formate hydrogenlyase activity. Indeed, it seems that the effects of the *oxrC* mutation on FDH-BV activity are more important in determining hydrogen evolution than are its effects on hydrogenase 3 activity since substantial hydrogenase 3 activity was detected under all conditions examined. However, it should be remembered that hydrogenase 3 activity may be due to two or more isoenzymes, only one of which is associated with formate hydrogenlyase.

The effects of the *oxrC* and *fnr* mutations on FDH-BV activity are very similar to their effects on the expression of an *fhl-lacZ* fusion (4, 7). The *fhl-lacZ* fusion used in these experiments lacked formate hydrogenlyase activity but still retained hydrogenase activity. Thus, the fusion was believed to be a fusion to the structural gene for FDH-BV. However, in the light of our findings that only one of the hydrogenase isoenzymes is required for formate hydrogenlyase activity, it was important to establish the isoenzyme contents of strains carrying this *fhl-lacZ* fusion. Direct immunoprecipitation showed that all three hydrogenase isoenzymes were present in normal amounts (data not shown). This is consistent with *fhl* being the structural locus for FDH-BV (4, 17, 18), and it also eliminates the possibility that the effects of *oxrC* and *fnr* mutations on the hydrogenase isoenzymes are mediated via the *fhl* locus. The *tppR* mutation (7) had no effect on FDH-BV activity, as anticipated from its lack of effect on *fhl* expression (7).

In addition to FDH-BV, the effects of *oxrC* and *fnr* mutations on other anaerobically induced enzymes were also examined (Table 4). Neither nitrate reductase nor fumarate reductase activity was affected by an *oxrC* mutation. However, as anticipated from results with *E. coli* (10, 16), both activities were drastically reduced in *fnr* strains.

Phenotypic suppression of the *oxrC* mutation. We have shown that the *oxrC* mutation can be phenotypically suppressed by growth on fructose (7). Normal hydrogen metabolism was found for an *oxrC* strain after growth on fructose rather than on glucose (Table 5). Both hydrogen uptake during fermentative growth and isoenzyme 1 levels were restored to wild-type levels. Similarly, hydrogen evolution and the activities of the enzymes of the formate hydrogenlyase pathway (hydrogenase 3 and FDH-BV) were also fully restored by growth on fructose.

Hydrogen metabolism and catabolite repression. Since the hydrogenase isoenzymes were differentially expressed during fermentative and nonfermentative growth, it seemed likely that expression of the isoenzymes might be controlled by catabolite repression. Strains were constructed carrying *cya* and *crp* mutations, and hydrogenase activities were determined for cells grown fermentatively (glucose) in the presence or absence of added cyclic AMP (cAMP) (Table 6). Two major effects were observed. First, hydrogenase isoenzyme 2 was found to be subject to catabolite repression. The cellular content of this isoenzyme was enhanced by the addition of cAMP, both in the wild-type and in the *cya* strain (Fig. 2c and d). This enhancement was not seen in the *crp* strain (Fig. 2e and f), showing the cAMP effect to be mediated by the cAMP receptor protein (CRP). However, it should be noted that there was always a substantial basal level of isoenzyme 2 expression which was maintained independently of the catabolite repression system. The repression of isoenzyme 2 during fermentative (glucose) growth is, of course, compatible with the respiratory role of this enzyme.

The second important effect was that added cAMP was found to reduce formate hydrogenlyase activity, particularly

TABLE 5. Suppression of the *oxrC* effect on hydrogen metabolism by fermentative growth on fructose

Strain and growth conditions[a]	Sp act (U/mg of protein) of:				
	Formate hydrogenlyase[b]	Hydrogen uptake[b]	Hydrogenase[c]		
			Isoenzyme 1	Isoenzyme 2	Nonimmunoprecipitable activity
LT2 (Z) with glucose	0.207	0.042	0.079	0.811	0.546
LT2 (Z) with fructose	0.183	0.035	0.046	0.968	0.703
CH1021 (*oxrC*) with glucose	0.067	0.002	0.008	1.05	0.272
CH1021 (*oxrC*) with fructose	0.282	0.052	0.123	0.706	1.01

[a] Cells were harvested at the late exponential phase of growth, washed once, and resuspended in 100 mM potassium phosphate (pH 6.8). The assays on the whole cells were performed immediately. Membrane fractions were prepared as described in Materials and Methods.

[b] Assays performed on whole cells.

[c] Expressed per milligram of protein in the Triton X-100-dispersed membrane fractions. Isoenzyme 1, isoenzyme 2, and nonimmunoprecipitable hydrogenase activities were calculated as described in Materials and Methods and in Table 3.

in a *cya* mutant. Curiously, the addition of cAMP to the growth medium of wild-type cells did not fully mimic the effects seen for the *cya* mutant; we have no explanation for this at present. Again, the cAMP effects were dependent upon CRP, since cAMP had no effect in a *crp* strain. The effects of cAMP on hydrogenase isoenzyme 3 paralleled its effects on formate hydrogenlyase activity, consistent with this isoenzyme being associated with the formate hydrogenlyase pathway and with its fermentative role. As for isoenzyme 3, the levels of isoenzyme 1 were also reduced by the addition of cAMP (Fig. 2a and b). However, *cya* and *crp* mutations had complex effects on the expression of this isoenzyme, which are not fully understood at present. Thus, it is clear that expression of the individual hydrogenase isoenzymes is regulated by the cAMP-CRP system in a manner consistent with their fermentative or nonfermentative roles.

DISCUSSION

In this paper we describe in detail the regulation of hydrogen metabolism in *S. typhimurium*, both by anaerobic regulatory mutations and in response to different growth conditions. An examination of the specific regulation of each of the individual hydrogenase isoenzymes has provided strong support for the proposed functions of each of the isoenzymes in hydrogen metabolism and, in addition, defines the physiologically distinct nature of the *oxrC*- and *fnr*-dependent anaerobic regulatory pathways.

The *oxrC* and *fnr* mutations define pleiotropic anaerobic regulatory pathways, which we have suggested may serve distinct physiological roles (7). Because the hydrogenase isoenzymes serve both fermentative and respiratory roles, this seemed like an ideal system to examine such a hypothesis. Both *fnr* and *oxrC* mutations were found to have distinct effects on expression of different hydrogenase isoenzymes. Isoenzyme 2 was found to be *fnr* dependent and *oxrC* independent, whereas, conversely, isoenzyme 3 was *fnr* independent, but both activity and formate inducibility were considerably reduced in an *oxrC* strain. In addition to regulating the expression of the hydrogenase component of formate hydrogenlyase (hydrogenase 3), *oxrC* mutants also have reduced levels of the second enzyme of the system, FDH-BV. This effect is transcriptional, as shown by the use of an *fhl-lacZ* fusion. These observations are entirely consistent with the effects of the *fnr* and *oxrC* mutations on hydrogen metabolism (respiratory hydrogen uptake is *fnr* dependent, whereas fermentative hydrogen evolution is *oxrC* dependent) and thus provide strong support for a role for isoenzyme 2 in hydrogen uptake and for isoenzyme 3 in hydrogen evolution catalyzed by formate hydrogenlyase.

TABLE 6. Hydrogen metabolism in mutants defective in catabolite repression

Strain	Addition to growth medium[a]	Sp act (U/mg of protein) of:	
		Formate hydrogenlyase[b]	Nonimmunoprecipitable activity[c]
LT2 (Z)	None	1.0	0.84
LT2 (Z)	cAMP	0.79	0.49
CH1107 (*cya*)	None	1.30	0.25
CH1107 (*cya*)	cAMP	0.18	<0.02
CH1108 (*crp*)	None	1.73	0.54
CH1108 (*crp*)	cAMP	1.70	0.36

[a] Growth medium consisted of NB and glucose as described in Materials and Methods and cAMP (5 mM) as indicated. Cells were harvested and immediately subjected to the intact-cell assays as described in Materials and Methods.

[b] Assays performed on whole cells.

[c] Expressed as the specific activity in Triton X-100-dispersed membrane fractions, for the hydrogenase activity that was not immunoprecipitated by antibodies specific for either hydrogenase isoenzyme 1 or 2 (see Materials and Methods).

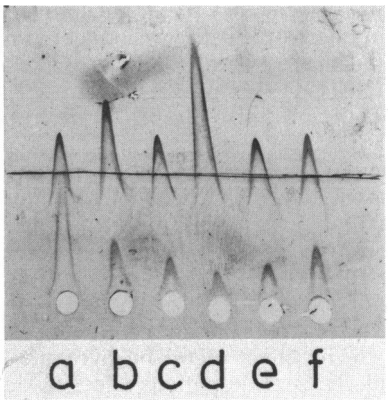

FIG. 2. Hydrogenase isoenzyme 1 and 2 antigen contents of strains defective in catabolite repression. Triton X-100-dispersed membrane fractions (40 μg of protein) were analyzed by rocket immunoelectrophoresis on a divided immunoplate using antibodies to *E. coli* hydrogenase isoenzyme 2 (top layer) and to *E. coli* hydrogenase isoenzyme 1 (bottom layer). All strains were grown anaerobically in NB containing glucose and cAMP (5 mM) as indicated. Samples are from the same experiment described in Table 6. Lanes, a, LT2 (Z); b, as for lane a, but grown with cAMP; c, CH1107 (*cya*); d, as for lane c, but grown with cAMP; e, CH1108 (*crp*); f, as for lane e, but grown with cAMP.

Hydrogenase isoenzyme 1 is, unusually, dependent on both *fnr* and *oxrC*. Previously (24), we proposed that isoenzyme 1 may serve a role in hydrogen recycling. We envision that hydrogen produced by formate hydrogenlyase activity during fermentative growth is recaptured by isoenzyme 1 and used to reduce endogenously synthesized electron acceptors. This isoenzyme therefore serves both fermentative and respiratory roles.

The above results provide good evidence for the pleiotropic nature of the *oxrC*-dependent anaerobic pathway and its distinction from the *fnr*-dependent pathway. In addition, the results provide excellent support for the suggestion (7) that *fnr* and *oxrC* define two distinct anaerobic regulatory pathways with different physiological roles; *fnr*-dependent enzymes serve essentially respiratory functions, whereas *oxrC*-dependent enzymes serve fermentative or biosynthetic roles. Thus, the fermentative hydrogenase (isoenzyme 3) is *oxrC* dependent, whereas the respiratory hydrogenase (isoenzyme 1) is *fnr* dependent. Significantly, isoenzyme 1, which serves both a respiratory and a fermentative role, is dependent on both *fnr* and *oxrC*. Why should transcription of anaerobically induced genes whose products serve fermentative roles be under a separate control system to that of genes with respiratory functions? The most probable explanation is that, although respiratory proteins are normally only required under very specific conditions (for example, anaerobically in the presence of the appropriate electron acceptor), the transcription of genes encoding fermentative enzymes is required at a basal level at all times but must be increased to higher levels under specific conditions (e.g., anaerobiosis in the absence of an electron acceptor). In addition, enzymes like formate hydrogenlyase are required at high levels anaerobically only in the absence of respira-

tion. Our data suggest that the fermentative enzymes are regulated by fermentative pathways (glycolysis), while it has been suggested that respiratory enzymes are regulated by redox potential (19). This provides a further, and logical, distinction between the two classes of anaerobically inducible genes.

Despite the demonstrated distinction in the physiological roles of the *oxrC*- and *fnr*-dependent regulatory pathways, both systems interact, albeit indirectly, at the level of formate. For example, *fnr* mutations exert some effect on *fhl* activity. This effect is indirect, as activity is fully restored by the addition of exogenous formate, and is interpreted as being due to a reduction in the synthesis of endogenous formate due to the effect of *fnr* on pyruvate formatelyase activity. Similarly, *oxrC* mutations affect *fhl* activity in two ways; in addition to the direct effect on anaerobic induction, there is a secondary effect prevented by the addition of exogenous formate. This is again indirect, being due to the metabolic effects of *oxrC* mutations, which almost certainly reduce endogenous formate synthesis. We in no way intend to imply that formate serves as an anaerobic signal molecule.

Because the various hydrogenase isoenzymes serve either fermentative or respiratory roles, a role for catabolite repression in the regulation of the cellular isoenzyme content might be anticipated. In addition to the anaerobic regulation of hydrogenase function, we found that hydrogen metabolism is subject to catabolite repression in a complex manner. Consistent with its respiratory role, isoenzyme 2 expression is repressed by glucose in a CRP-dependent manner. However, even in a *crp* mutant there is still a significant basal level of isoenzyme 2 expression; this is in contrast to *crp*-dependent regulation at the *lac* promoter but similar to that found for the *gal* operon (26). Formate hydrogenlyase activity and its associated hydrogenase (hydrogenase 3) are apparently regulated in the opposite manner to hydrogenase 2, being inhibited by the addition of cAMP. Again, regulation is CRP dependent. This finding is, of course, consistent with the fermentative role of formate hydrogenlyase. This suggests that for this isoenzyme the CRP-cAMP complex may serve, unusually, to decrease gene expression. However, the situation is unclear, since in contrast to the *cya* mutant, there is no significant inhibition of formate hydrogenlyase activity by cAMP in a wild-type strain. We have no explanation at present for this unexpected behavior. Similarly, the expression of isoenzyme 1 also appears to be reduced by the cAMP-CRP complex.

Our data suggest that the CRP-cAMP complex may serve as an activator of expression of one isoenzyme (hydrogenase 2) and, unusually, as a repressor of another (hydrogenase 3). An inhibitory role for the CRP-cAMP complex was suggested previously (13). However, the mechanisms by which this is brought about require further clarification. Thus, the regulation of hydrogenase isoenzyme content by CRP and cAMP is consistent with the proposed physiological roles of the enzymes.

In addition to regulation by anaerobiosis and by the catabolite repression system, which presumably modify transcription of the *hyd* structural genes, the activity of all three hydrogenase isoenzymes can be modified by mutations at four additional loci, *hydA* and *hydB* (11, 21, 29), as well as *hydC* and *hydD* (30). Whereas nickel metabolism is implicated in the function of some of these loci (29, 30), the roles of the other pleiotropic *hyd* loci remain unclear, although they probably act at a posttranscriptional level. Thus, the regulation of hydrogen metabolism in enterobacteria is extremely complex. The cellular content of each of the hydrogenases is regulated in a distinct and very precise manner, such that each is synthesized only under growth conditions in which its activity is required.

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