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Ubiquinone Synthetic Pathway in Flagellation of *Salmonella typhimurium*

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Flagellation of *Salmonella typhimurium* was found to require a functional pathway for ubiquinone synthesis as well as growth in the presence of aliphatic or aromatic carboxylic acids. Selection for constitutive flagellation eliminated the requirement for growth in the presence of added carboxylic acids.

The regulation of flagellation is of interest in revealing patterns of differentiation control. Experiments reported previously have pointed out the possible modulation of flagellation by means of growth phase (15), temperature (12, 14), nucleotide and amino acid analogs (13), nutrient type (12, 18), and cAMP (20).

Aerobic flagellation in *Escherichia coli* was reported recently to require ubiquinone (4, 9). The ubiquinone-flagellation relationship was further pursued here by analyzing flagellation of mutants in which the ubiquinone content was manipulated in situ by growth in the presence of variable concentrations of *p*-hydroxybenzoic acid. Flagellation induced by *p*-hydroxybenzoic acid was found to reflect (i) a functional pathway for ubiquinone synthesis requiring a relatively low *p*-hydroxybenzoate concentration and (ii) induction of flagellation by specific carboxylic acids requiring a relatively high *p*-hydroxybenzoate concentration. Selection for constitutive flagellation was found to eliminate the requirement for carboxylate-induced flagellation.

MATERIALS AND METHODS

Salmonella typhimurium LT2, *S. typhimurium* 1120 (*aroD5*), and *S. typhimurium* TA1851 (*hisT5670* (3) were obtained from G. Ames. *S. typhimurium* ST1 (2) was selected from *S. typhimurium* LT2 for optimal chemotaxis to serine on semisolid minimal agar plates (2).

Bacteria were grown aerobically to midlog phase in VB minimal medium (19) supplemented with 5 mM citrate, 0.5% glycerol, and additions as described. The growth medium for *S. typhimurium* 1120 (*aroD5*) was supplemented with 0.2 mM L-tryptophan, 0.2 mM L-phenylalanine, and 0.02 mM purified L-tyrosine. L-tyrosine was rid of *p*-hydroxybenzoic acid contamination by repeated extractions of a 2.0 mM acidic aqueous solution (pH 2.0) with an equal volume of ether. The extracted aqueous solution was neutralized, and the concentration was determined spectrophotometrically. All incubations were carried out at 30°C with continuous gyratory shaking.

Ubiquinone content and respiration rate were determined as described previously (4). Uptake studies were performed with cultures which were harvested and washed three times with VB minimal medium supplemented with 0.01 mM EDTA. The washed pellet was suspended in the same medium and starved for 5 h at 30°C. For uptake measurements, glucose (22 mM) or succinate (20 mM) was added as a carbon source followed 10 min later by [^{14}C]glycine (100 μCi/μmol) or [^{14}C]glutamine (10 μCi/μmol) to a final concentration of 10 μM. Samples of the incubation mixture were removed every 8 s after addition of the radioactive amino acid over a period of 1 min onto individual 0.45-μm membrane filters (HA; Millipore Corp.). The filters were washed three times with 5 ml of VB minimal medium supplemented with 0.01 mM EDTA, dried at 110°C for 5 min, and counted in Aquasol scintillation fluid.

The extent of flagellation as a function of the strain used or of additions to the growth medium was determined by the motility photographic assay (4), by the chemotactic response (1), or by screening the number of flagella per bacterium as described previously (4). Alterations in medium composition did not affect the motility percentage nor the chemotactic response of bacteria having well-developed flagella. Motile cultures were defined as those having more than 70% motile bacteria, whereas cultures having less than 1% motile bacteria were defined as nonmotile. The 30-min chemotactic response to a concentration change of 0 to 10 mM serine was measured by the capillary assay at 30°C as described by Adler (1) with a bacterial suspension of 5×10^6 cells/ml.

RESULTS

Ubiquinone was shown recently to be obligatory for aerobic flagellation in *E. coli* (4, 9). This observation has initiated the search for appropriate mutants in ubiquinone synthesis for quantitative analysis of the ubiquinone-flagellation relationship. *S. typhimurium* TA1851 and *S. typhimurium* 1120 (*aroD5*), derived independently from wild-type *S. typhimurium* LT2, were found suitable for this study since both depend on the presence of *p*-hydroxybenzoate for biosynthesis

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of the wild-type complement of ubiquinone. Whereas the *aroD5* mutant is an aromatic amino acid as well as *p*-hydroxybenzoate auxotroph, being mutated in chorismate synthetase (17), the TA1851 strain, derived as a *his* deletion mutant (3), is shown elsewhere (10) to be deficient in the decarboxylation step of polyprenyl *p*-hydroxybenzoate (6, 16) and to overcome this deficiency in the presence of *p*-hydroxybenzoate in the growth medium. The two *p*-hydroxybenzoate-dependent mutants were compared with *S. typhimurium* ST1 selected for optimal chemotaxis for serine (2) in the absence of added *p*-hydroxybenzoate. The ubiquinone content of *S. typhimurium* ST1 was not affected by *p*-hydroxybenzoate in the medium, and the native ubiquinone content approached the maximal attainable level, whereas the ubiquinone content of the two ubiquinone-deficient strains responded positively to *p*-hydroxybenzoate (Table 1). The increment in total ubiquinone was shown by reverse-phase thin-layer chromatography (7) to be distributed proportionally among the various polyprenyl ubiquinone species, with the major increase (90%) in octaprenyl ubiquinone. The motility pattern of the three strains as a function of *p*-hydroxybenzoate in the medium followed the ubiquinone response to added *p*-hydroxybenzoate. Thus, ST1 was motile irrespective of added *p*-hydroxybenzoate, whereas TA1851 and *aroD5* were essentially nonmotile after growth in the absence of *p*-hydroxybenzoate and became fully motile in its presence (Table 1). This observation was further corroborated by analyzing the average number of flagella of strain TA1851 and the *aroD5* mutant grown in the presence and absence of *p*-hydroxybenzoate (Table 2). Motility percentage was related to the percentage of bacteria with six or more flagella per bacterium for strain TA1851 and three or more flagella per bacterium for the *aroD5* mutant. In the absence of *p*-hydroxybenzoate, the number of flagella of the two mutant strains was significantly reduced.

TABLE 1. *Effect of p-hydroxybenzoic acid on ubiquinone content and motility of S. typhimurium strains*[a]

S. typhimurium strain	*p*-Hydroxybenzoate in growth medium	Motility	Ubiquinone (nmol/mg [dry wt] of bacteria)
TA1851	−	−	0.31
TA1851	+	+	0.73
1120 (*aroD5*)	−	−	0.03
1120 (*aroD5*)	+	+	0.83
ST1	−	+	0.78
ST1	+	+	0.77

[a] *S. typhimurium* strains TA1851, 1120 (*aroD5*), and ST1 were grown in VB minimal medium supplemented with 5 mM citrate and 0.5% glycerol in the absence or presence of 200 μM *p*-hydroxybenzoate. Ubiquinone content was determined as described previously (4).

The ubiquinone-flagellation relationship of strain TA1851 was further analyzed by determining the ubiquinone content and the motility percentage as a function of *p*-hydroxybenzoate concentration. The chemotactic response as a function of the ubiquinone content was used as an additional measure of the extent of flagellation. The motility percentage (Fig. 1A) and the chemotactic response (Fig. 1B) were both correlated with ubiquinone content. Hence, *p*-hydroxybenzoate-dependent flagellation could be ascribed to the role played by *p*-hydroxybenzoate as a precursor of ubiquinone. However, the following lines of evidence may point to an additional role of *p*-hydroxybenzoate in flagellation.

(i) The ubiquinone-deficient strain TA1851, though nonmotile, did not seem to be deficient in other properties related with oxidative electron transport. Thus, glucose or succinate respiration was not affected by the relatively low ubiquinone content (not shown). Similarly, glycine or glutamine uptake, coupled with oxidative or substrate-level phosphorylation, respectively (5), remained unaffected (not shown). Hence, the partial ubiquinone content of strain TA1851 (Table 1) appeared to be sufficient for the expression of functions related to oxidative electron transport, but still insufficient for flagellation.

(ii) The motility response to changes in ubiquinone level could be examined more closely by using the LT2 wild-type strain, in which the initial ubiquinone content was about 80 to 90% of that obtained in the presence of added *p*-hydroxybenzoate. The correlations between *p*-hydroxybenzoate and ubiquinone content, *p*-hydroxybenzoate and chemotaxis, and ubiquinone content and chemotaxis are presented in Fig. 2. As shown, the increase in motility response occurred within a narrow range of the increase in ubiquinone content. It is unlikely that an increase of 5% in ubiquinone content will result in flagellation unless an exceptionally cooperative effect is involved.

The motility percentage of LT2 cultures grown in the absence of added *p*-hydroxybenzoate was quite variable (0 to 25%). However, the *p*-hydroxybenzoate concentration that induced full motility of the strain LT2 was found to be unrelated to the initial motility observed in the absence of added *p*-hydroxybenzoate. Moreover, the motility pattern of strain LT2 was affected within the same concentration

TABLE 2. *p-Hydroxybenzoate-dependent flagellation of S. typhimurium TA1851 and 1120 (aroD5)*[a]

Bacteria and growth conditions	% of bacteria with given no. of flagella																	Mean no. of flagella/cell	Motility (%)
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
TA1851 (colony no. 7-19)	66	14	7	7	3	3	0	0	0	0	0	0	0	0	0	0	0	0.8	1
TA1851 (colony no. 7-19) + *p*-hydroxybenzoate	0	0	0	0	0	4	8	28	8	8	4	4	24	4	4	0	0	9.2	85
TA1851 (colony no. 8-6)	41	7	15	6	9	10	6	3	3	0	0	0	0	0	0	0	0	2.3	3
TA1851 (colony no. 8-6) + *p*-hydroxybenzoate	0	0	0	0	3	11	3	19	6	6	11	26	0	3	6	3	3	9.5	86
1120 (*aroD5*)	89	5	3	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0.2	0
1120 (*aroD5*) + *p*-hydroxybenzoate	20	17	17	27	10	7	3	0	0	0	0	0	0	0	0	0	0	2.2	55

[a] Cultures for electron microscopy were prepared as described previously (4). The number of flagella on each bacterium was recorded and counted. Motility was measured by the motility assay as described previously (4). *p*-Hydroxybenzoate was used at a concentration of 100 μM.

range of *p*-hydroxybenzoate as that which affected the motility response of strain TA1851, despite the difference in the initial ubiquinone content of the two strains.

(iii) The ubiquinone-motility correlation curves of the *aroD5* mutant as a function of added *p*-hydroxybenzoate (Fig. 3) indicate clearly that the maximal attainable level of ubiquinone did not seem to be high enough for *S. typhimurium* flagellation. Thus, motility was observed only at a *p*-hydroxybenzoate concentration range that was two to three orders of magnitude higher than the concentration of *p*-hydroxybenzoate required for restoration of the ubiquinone content in this strain. Hence, a novel role of *p*-hydroxybenzoate in flagellation should be considered in addition to its involvement in the ubiquinone final product.

To examine the dual role played by *p*-hydroxybenzoate in flagellation we looked for *p*-hydroxybenzoate analogs that would induce flagellation without serving as ubiquinone precursors. The motility pattern observed in nutrient broth indicated that *p*-hydroxybenzoate is not unique in generating motility of various *S. typhimurium* strains. Thus, *S. typhimurium* strains TA1851 and LT2 became motile upon culture in nutrient broth without being affected in ubiquinone content. Indeed, aliphatic as well as various aromatic carboxylic acids could substitute for *p*-hydroxybenzoate (Table 3). All saturated as well as certain unsaturated (e.g., oleic and palmitoleic) aliphatic carboxylic acids with an even carbon chain of C_4 through C_{18} were found to induce flagellum formation. Some substituted carboxylic acids were found to lack this ability (pyruvate, phenylpyruvate, α-amino acids), but a more conclusive definition of structural constraints must await further analysis. Furthermore, some of the compounds listed above could induce flagellation at a lower concentration range as compared with *p*-hydroxybenzoate. Thus, the apparent K_m values for hexanoate and benzoate in the growth medium were 5 and 10 μM, respectively, as compared with the apparent K_m value of 40 to 50 μM for *p*-hydroxybenzoate.

It should be pointed out that carboxylic acids other than *p*-hydroxybenzoate induce flagellation in a mutant like TA1851, which still contains enough of the ubiquinone synthetic pathway to sustain partial ubiquinone synthesis. However, carboxylates other than *p*-hydroxybenzoate will not induce flagellation in a mutant grown under conditions that eliminate the ubiquinone synthetic pathway. Thus, the *aroD5* mutant, being an auxotroph for *p*-hydroxybenzoate, was barely motile in the presence of added hexanoate. However, flagellation was expressed in the presence of hexanoate added together with trace amounts of *p*-hydroxybenzoate (Fig. 4). The concentration range for *p*-hydroxybenzoate that allowed for flagellation in the presence of a carboxylate (Fig. 4) was three orders of magnitude lower than the *p*-hydroxybenzoate concentration required for flagellation in the absence of added carboxylate (Fig. 3). Actually, the *p*-hydroxybenzoate concentration that allowed for flagellation in the presence of a carboxylate was within the range for *p*-hydroxybenzoate acting as a precursor for ubiquinone synthesis (Fig. 4). Hence, reconstitution of flagellation of the *aroD5* mutant required growth either in the presence of a relatively high *p*-hydroxybenzoate concentration (in the range of 100 to 500 μM) or in the presence of added carboxylic acids together with trace amounts of *p*-hydroxybenzoate. This heterotropic combination will allow for ubiquinone synthesis as well as flagellation-inducing ability and thus may substitute for the flagellation-inducing concentration of *p*-hydroxybenzoate that maintains both functions.

The growth yield of mutant TA1851 in glycerol, pyruvate, or succinate minimal medium was low in the absence of added *p*-hydroxybenzoate. However, carboxylate-induced flagella-

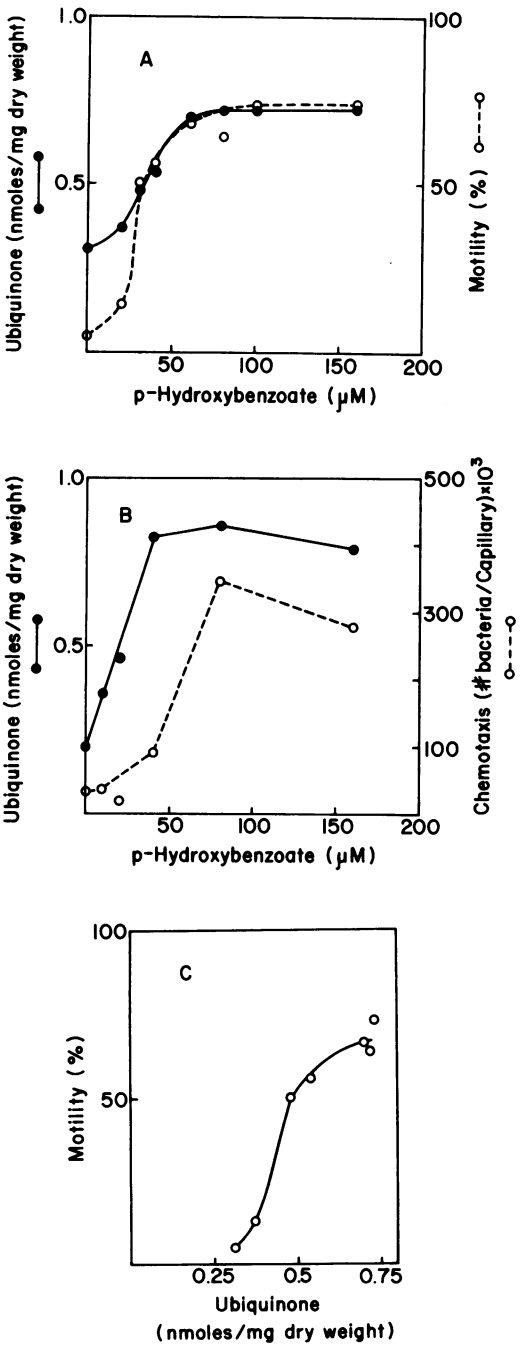

FIG. 1. *Ubiquinone content, motility percentage, and chemotaxis of S. typhimurium strain 1851 as function of added p-hydroxybenzoate. Strain TA1851 was grown to midlog phase in VB minimal medium supplemented with 5 mM citrate and 0.5% glycerol with increasing concentrations of p-hydroxybenzoate. Ubiquinone content, motility percentage, and the 30-min chemotactic response to a concentration change of 0 to 1.0 mM L-serine were measured as described previously (4). (A) p-Hydroxybenzoate-dependent motility and ubiquinone synthesis; (B) p-hydroxybenzoate-dependent chemotaxis and ubiquinone synthesis; (C) ubiquinone-dependent motility.*

tion cannot be ascribed to differences in growth yields, since the rapidly growing cells in glucose culture were nonmotile, whereas the rapidly growing cells in *p*-hydroxybenzoate culture were motile. Nonmotility in the glucose culture cannot be ascribed to glucose catabolite repression of flagellation since the *p*-hydroxybenzoate-grown culture was shown to escape catabolite repression in glucose minimal medium. Moreover, the benzoate-induced culture was motile without being affected in its generation time. Similarly, *S. typhimurium* LT2 had the same doubling time (80 min) in glycerol minimal medium in the presence or absence of *p*-hydroxybenzoate, but flagellation was expressed only with the *p*-hydroxybenzoate-grown culture.

The time dependence of *p*-hydroxybenzoate-induced flagellation of strain LT2 is shown in Fig. 5. In the presence of *p*-hydroxybenzoate, motility was induced gradually over a time period of five to seven generations. Similarly, *p*-hydroxybenzoate-induced motility of strains LT2 and TA1851 was lost over five to seven generations when *p*-hydroxybenzoate was omitted from the growth medium of preinduced cultures. Similar observations were obtained with both hexanoate- and benzoate-induced cultures.

DISCUSSION

Induction of flagellation in *S. typhimurium* has been shown here to require a functional pathway for ubiquinone synthesis as well as growth in the presence of appropriate carboxylates (Table 4). Thus, the *aroD5* mutant, which lacks a functional pathway for ubiquinone synthesis when grown in the absence of *p*-hydroxybenzoate, will not become flagellated unless induced by an appropriate carboxylic acid under conditions that allow for ubiquinone synthesis. Since both conditions must be met simultaneously, the *aroD5* mutant will become flagellated in the presence of a carboxylate other than *p*-hydroxybenzoate together with trace amounts of *p*-hydroxybenzoate as a precursor for the ubiquinone synthetic pathway. Alternatively, since *p*-hydroxybenzoate may serve as a carboxylate inducer, and since the required inductive concentration is much higher than the concentration required for *p*-hydroxybenzoate as a precursor of ubiquinone, growth in the presence of an inductive concentration of *p*-hydroxybenzoate

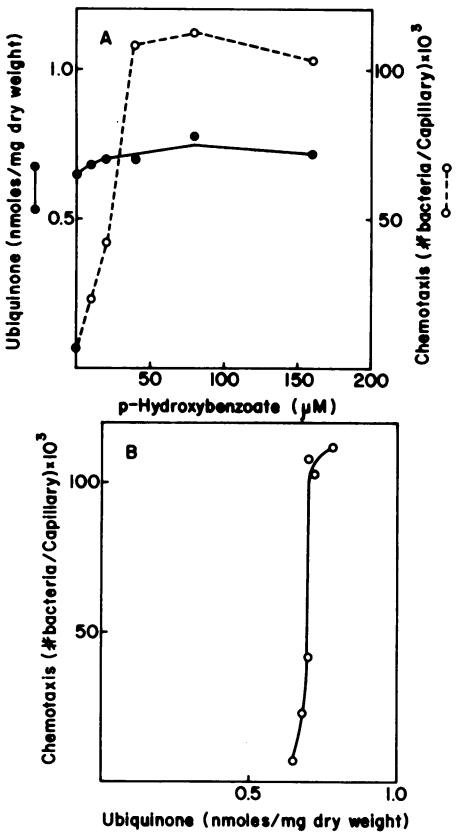

FIG. 2. *Ubiquinone content and chemotaxis of S. typhimurium strain LT2 as function of added p-hydroxybenzoate. Strain LT2 was grown to midlog phase in VB minimal medium supplemented with 5 mM citrate and 0.5% glycerol with increasing concentrations of p-hydroxybenzoate. Ubiquinone content and the chemotactic response to a concentration change of 0 to 1.0 mM L-serine were determined as described previously (4). (A) p-Hydroxybenzoate-dependent chemotaxis and ubiquinone synthesis; (B) ubiquinone-dependent chemotaxis.*

will induce flagellation. In contrast to the *aroD5* mutant, the wild-type LT2 strain has a functional ubiquinone synthetic pathway in the absence of added *p*-hydroxybenzoate. Hence, growth in the presence of a suitable carboxylate fulfills the sole condition for flagellation, eliminating the specific requirement for added *p*-hydroxybenzoate. The TA1851 strain should be regarded in this context as similar to the LT2 strain. In spite of its partial complement of ubiquinone as compared with LT2, the available ubiquinone pathway of the TA1851 strain is sufficient for flagellation in the absence of added *p*-hydroxybenzoate, and flagellation is induced by any appropriate carboxylate inducer. In contrast to the wild-type LT2 strain, the ST1 strain, selected for motility in minimal medium, has a functional ubiquinone synthetic pathway as well as flagellation in the absence of added carboxylate and may be regarded as constitutive in this context.

The requirement for a functional ubiquinone synthetic pathway may reflect either the role played by an intermediate of the pathway in flagellation or the requirement for the final ubi-

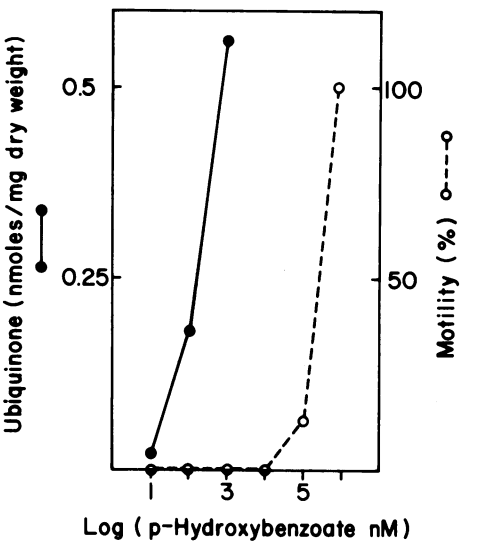

FIG. 3. *p-Hydroxybenzoate-dependent ubiquinone content and motility percentage of S. typhimurium mutant aroD5. Mutant aroD5 was grown as described in the text with increasing concentrations of p-hydroxybenzoate in the growth medium. Ubiquinone content and motility percentage were determined as described previously (4).*

TABLE 3. *Motility induction of S. typhimurium TA1851 by carboxylic acids*[a]

Addition	Motility (%)	Ubiquinone content (nmol/mg [dry wt])
None	0	0.52
Triton X-100 (1%)	0	0.50
Benzoate (200 μM)	82	0.40
Hexanoate (200 μM) in Triton X-100 (1%)	75	0.51
Octanoate (200 μM) in Triton X-100 (1%)	75	0.50
Palmitate (200 μM) in Triton X-100 (1%)	75	0.51
p-Hydroxybenzoate (100 μM)	80	0.77

[a] *S. typhimurium* TA1851 was grown to midlog phase in VB minimal medium supplemented with 5 mM citrate and 0.5% glycerol with additions as described. The saturated aliphatic acids used are examples taken from the complete C_4-C_{18}, all of which were tested. Ubiquinone content and motility percentage were determined as described previously (4).

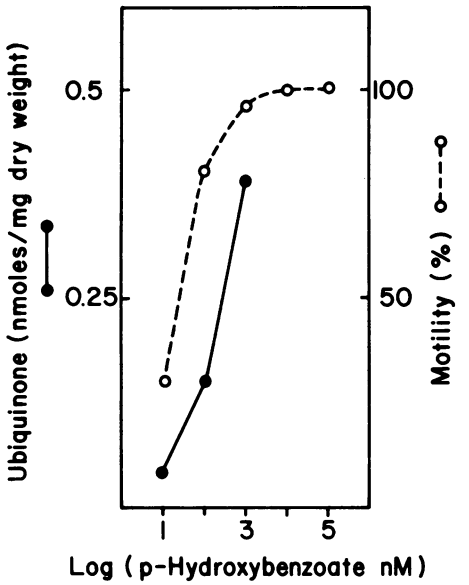

FIG. 4. *p-Hydroxybenzoate-dependent ubiquinone content and motility percentage of S. typhimurium mutant aroD5 in the presence of hexanoate. Mutant aroD5 was grown as described in the text in the presence of 400 μM hexanoate and increasing concentrations of p-hydroxybenzoate. Ubiquinone content and motility percentage were determined as described previously (4).*

quinone product. The two possibilities are not mutually exclusive. The assumed requirement for the final ubiquinone product stems from observations reported previously which have pointed to the obligatory role played by aerobic electron transport in aerobic flagellation in *E. coli* (4, 9). Oxidative electron transport will be shown elsewhere to regulate the promotion of aerobic flagellin transcription (R. Hertz and J. Bar-Tana, manuscript in preparation). Ubiquinone as an obligatory component of oxidative electron transport is therefore involved in flagellation, whether induced or constitutive. However, apart from the requirement for the ubiquinone product, an intermediate of the ubiquinone synthetic pathway is shown elsewhere to be implicated in carboxylate-mediated flagellation (10). This intermediate is shown to be related with the decarboxylation step of polyprenyl *p*-hydroxybenzoate to polyprenylphenol (6, 10, 16). Thus, the ubiquinone synthetic pathway seems to play a dual role in flagellation through an intermediate of the pathway as well as through the final ubiquinone product.

The considerable time lag which characterizes carboxylate-induced flagellation as reported here cannot be ascribed to the gradual selection of a motile preexisting subpopulation. Possible selection of a supposedly preexisting motile subpopulation with concomitant selection against an initially nonmotile subpopulation would require a differential growth rate for the two assumed subpopulations. Thus, a doubling time of 56 min for the supposedly motile subpopulation could be calculated from Fig. 5 by multiplying the observed motility percentage and the respective optical density of the *p*-hydroxybenzoate growing culture. Similarly, a doubling time of 106 min for the supposedly nonmotile subpopulation could be calculated from Fig. 5 by multiplying the observed nonmotility percentage and the respective optical density of the culture grown in the absence of *p*-hydroxybenzoate. However, these figures are not supported by the observed LT2 doubling time of 80 min, which remained unaffected by the presence or absence in the growth medium of *p*-hydroxybenzoate. Similarly, the induction of flagellation observed here was shown not to involve transformation of an O-H phase variation culture into an H_1-H_2 phase variation (11) (J. Bar-Tana and L. Lobel, manuscript in preparation). Hence, motility induction by carboxylic acids seems to reflect a novel phenomenon related to the flagellation apparatus per se. The experimental procedures used here cannot distinguish flagellin synthesis, flagellin polymerization, or flagella assembly, and the observation described here may involve

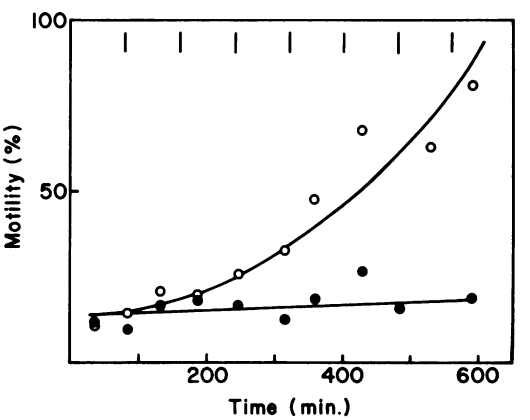

FIG. 5. *Time course of p-hydroxybenzoate-dependent flagellation in S. typhimurium strain LT2. Strain LT2 was grown to midlog phase in VB minimal medium supplemented with 5 mM citrate and 0.5% glycerol and was subsequently diluted in the absence* (○) *and presence* (●) *of 100 μM p-hydroxybenzoate. Growth yield was measured by following the increase in optical density at 650 nm. Motility was followed as described previously (4). The cultures were kept at midlog phase by appropriate dilution with the respective media. Bars indicate doubling time for both cultures (80 min).*

TABLE 4. *Flagellation as a function of a functional ubiquinone synthetic pathway and carboxylate induction in S. typhimurium*

S. typhimurium strain	Correlation under given growth conditions									
	−*p*-Hydroxybenzoate − carboxylate inducer		−*p*-Hydroxybenzoate + carboxylate inducer		+*p*-Hydroxybenzoate (0.1 μM) − carboxylate inducer		+*p*-Hydroxybenzoate (100 μM) − carboxylate inducer		+*p*-Hydroxybenzoate (0.1 μM) + carboxylate inducer	
	Flagellation	Ubiquinone content	Flagellation	Ubiquinone content	Flagellation	Ubiquinone content	Flagellation	Ubiquinone content	Flagellation	Ubiquinone content
1120 (*aroD5*)	−	−	−	−	−	++	+	++	+	++
LT2	−	++	+	++	−	++	+	++	+	++
TA1851 (*hisT5670*)	−	+	+	+	−	+	+	++	+	+
ST1	+	++	+	++	+	++	+	++	+	++

each one of the steps that contribute to flagellation.

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