JOURNAL OF BACTERIOLOGY, Apr. 2003, p. 2243–2250 Vol. 185, No. 7
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.7.2243–2250.2003

The Ascorbate Transporter of *Escherichia coli*

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Received 21 October 2002/Accepted 16 January 2003

The *sgaTBA* genes of *Escherichia coli* encode a putative 12-transmembrane α-helical segment (12 TMS) transporter, an enzyme IIB-like protein and an enzyme IIA-like protein of the phosphotransferase system (PTS), respectively. We show that all three proteins as well as the energy-coupling PTS proteins, enzyme I and HPr, are required for the anaerobic utilization and uptake of L-ascorbate in vivo and its phosphoenolpyruvate-dependent phosphorylation in vitro. The transporter exhibits an apparent K_m for L-ascorbate of 9 μM and is highly specific. The *sgaTBA* genes are regulated at the transcriptional level by the *yjfQ* gene product, as well as by Crp and Fnr. The *yjfR* gene product is essential for L-ascorbate utilization and probably encodes a cytoplasmic L-ascorbate 6-phosphate lactonase. We conclude that SgaT represents a novel prototypical enzyme IIC that functions with SgaA and SgaB to allow phosphoryl transfer from HPr(his-P) to L-ascorbate via the phosphoryl transfer pathway: PEP → enzyme I-P → HPr-P → IIA-P $\xrightarrow{\text{SgaA}}$ IIB-P $\xrightarrow{\text{SgaB}}$ IIC $\xrightarrow{\text{SgaT}}$ L-ascorbate-6-P.

In 1996, we reported computational analyses of three operons present in the *Escherichia coli* genome that appeared to encode enzymes and a transporter concerned with sugar metabolism (18). These operons, *sga*, *sgb*, and *sgc*, encode enzymes homologous to pentose-phosphate 3- and 4-epimerases, and *sga* and *sgc* also encode homologues of constituents of the bacterial phosphotransferase system (PTS) (22). The *sga* operon includes two genes, *sgaA* and *sgaB*, that encode homologues of fructose/mannitol enzymes IIA and lactose/*N*,*N*′-diacetylchitobiose enzymes IIB, respectively (Fig. 1). No IIC homologue was identified, but upstream of the *sgaB* and *sgaA* genes is a gene, *sgaT*, that encodes a membrane protein with 12 putative transmembrane helical segments, a characteristic of many secondary carriers (25). SgaT proved not to be homologous to any functionally characterized protein. Two possibilities were considered: (i) SgaT-SgaB-SgaA could be a novel type of enzyme II complex, or (ii) SgaT could be a secondary transporter, while SgaA and SgaB might regulate expression of the *sga* operon or the activity of one or more of its gene products (18, 25).

It has been known for over 60 years that various bacteria, including *E. coli*, can ferment L-ascorbate (L-xyloascorbate [vitamin C]) under anaerobic but not aerobic conditions (7, 19, 32). The unstable hydrolysis product of L-ascorbate, 3-keto-L-gulonate, has been implicated as an intermediate in its catabolism (28). The dissimilation of L-ascorbate, both during coculture with other carbon sources and as a sole carbon source, has been documented for *E. coli* (19, 31). In animal tissues, the principal route of L-ascorbate metabolism involves enzymatic and nonenzymatic oxidation to dehydroascorbate, an unstable lactone that hydrolyzes spontaneously to 2,3-di-keto-L-gulonate (9).

Recently, Yew and Gerlt (31) showed that the anaerobic utilization of L-ascorbate is dependent on three enzymes encoded downstream of *sgaTBA* within the *sga* operon (Fig. 1). These enzymes catalyze the conversion of the phosphorylated hydrolytic product of L-ascorbate, 3-keto-L-gulonate-6-P, to D-xylulose-5-P. They proposed that L-ascorbate or 3-keto-L-gulonate is phosphorylated by the PTS and that, subsequently, 3-keto-L-gulonate-6-phosphate decarboxylase (SgaH; renamed UlaD) produces L-xylulose-5-P (30), which is then converted to D-xylulose-5-P in a two-step process catalyzed by SgaU (renamed UlaE) and SgaE (renamed UlaF). Involvement of the putative permease SgaT (renamed UlaA by Yew and Gerlt [31]) and the PTS protein homologues SgaA (renamed UlaC) and SgaB (renamed UlaB) was not examined. Nor was it established whether L-ascorbate is hydrolyzed extracellularly or intracellularly. Consequently, it was not clear what the transported substrate should be.

Prior to the report of Yew and Gerlt (31), we had been taking a functional genomic approach to characterize the *sgaTBA* gene cluster. We had knocked out the three genes individually and together, but a PTS-related phenotype was not apparent. Using GN Biolog plates (2), we found that the *sgaTBA* deletion mutant seemed to oxidize L-proline less efficiently than the isogenic wild type, but used glycyl-L-glutamate somewhat better. Ascorbate was not included in any of the 96-well Biolog plates (2). The report of Yew and Gerlt (31) led us to test our mutants for defects in L-ascorbate utilization, transport, and phosphorylation. The results are presented in this report.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strains and plasmids used in this study are listed in Table 1. All studies were conducted in the genetic background of strain BW25113 (5), except for the analyses of the dependency of ascorbate utilization and transport on the energy-coupling proteins of the PTS, enzyme I and HPr. These studies were conducted in the genetic background of JM101 with strain PB11 (Table 1) (8).

Deletion mutants were generated by the methods described by Datsenko and Wanner (5). To prepare competent cells for transformation, BW25113 contain-

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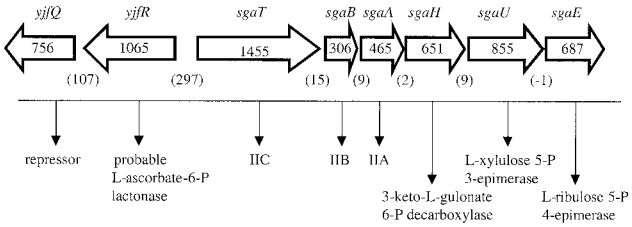

FIG. 1. The *E. coli sga* operon showing the gene names (above the genes, indicated by arrows) and gene lengths (number of base pairs within the arrows). The arrows, with lengths proportional to gene size, indicate the direction of transcription. The (putative) functions of the gene products are indicated below the arrows. The numbers in parentheses indicate the numbers of base pairs in the intergenic regions.

ing pKD46 was cultured at 30°C in SOB broth (24) containing 100 μg of ampicillin per ml. When the optical density at 600 nm (OD_{600}) reached 0.5, the culture was centrifuged at 4000 rpm for 5 min, and the cells were washed three times with cold 10% glycerol before being resuspended in a minimal volume of 10% glycerol (1% of the original culture volume). The competent cells were stored at −80°C prior to use. PCR methods were used to clone the kanamycin resistance gene (*km*) from pKD4 by using primers described in the supplementary table, Table S1, on our web site (www-biology.ucsd.edu/~msaier/supmat). These primers are available upon request. The PCR products were purified with a Qiagen kit, treated with *Dpn*I, and repurified by electrophoresis. The *km* gene was transformed into BW25113-competent cells by electroporation (Gene Pulser; pulse controller at 200 Ω, capacitance at 250 μF, and voltage at 25 kV). After electroporation, the cells were grown with shaking in 1 ml of SOC medium (24) at 37°C for 1 h, and the cultures were plated onto Luria-Bertani (LB) agar containing 25 μg of kanamycin per ml. The Km^r transformants were purified on new kanamycin-LB plates. The mutants in which the target genes were replaced by the *km* gene were verified by PCR with the pairs of primers listed in Table S1 on our web site.

To delete the *km* gene from the chromosome, pKD46 was removed from the cells by growing the bacteria at 37°C, and then pCP20, expressing the FLP recombinase, was introduced by transformation. The transformants containing pCP20 were grown overnight with shaking at 42°C, and the culture was plated on LB agar without antibiotics. Colonies were tested for sensitivity to kanamycin and ampicillin.

Growth conditions. Bacteria were cultured in LB complex medium or M9 minimal medium at 37°C (24). When appropriate, ampicillin and/or kanamycin was added to the medium at 100 and 25 μg/ml, respectively. To measure growth on L-ascorbate, bacteria were grown anaerobically in medium containing L-ascorbate as described by Yew and Gerlt (31). Briefly, *E. coli* strains were grown overnight on LB agar, and the cells were suspended in M9 salts medium. The OD_{600} of the cell suspension was adjusted to 1.0, and 100-μl aliquots were inoculated into 10-ml screw-cap culture tubes (Fischer Scientific) that were filled to the top with M9 medium plus one or more carbon sources, each at a concentration of 20 mM unless otherwise specified. The tubes were capped, sealed with parafilm, and then incubated at 37°C. The cell density during growth was measured with a Klett photoelectric colorimeter. To culture strains JM101 (wild type) and PB11 (*ΔptsHIcrr*) in M9 medium, thiamine was added to the medium to a final concentration of 40 μg/liter. We tested the effect of cyclic AMP on the growth of strain PB11 by using L-ascorbate as the sole source of carbon, with neutralized cyclic AMP added to the growth medium at concentrations between 0.25 and 2.5 mM. To culture bacterial strains containing plasmid pBAD24 with or without a cloned gene, ampicillin and 2 mM L-arabinose were added to the medium.

DNA manipulations and gene cloning. Standard methods were used for chromosomal DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (24). Plasmids were isolated by using spin miniprep kits (Qiagen, Chatsworth, Calif.), and PCR products were purified with Qiaquick purification kits (Qiagen). For gene cloning, the *sgaTBA*, *sgaT*, *sgaB*, *sgaA*, *yjfQ*, and *yjfR* genes were amplified from chromosomal DNA of wild-type *E. coli* strain BW25113 by PCR. The primers used for gene amplification are listed in the supplementary table on our web site, Table S2 (restriction sites *Xba*I and *Hin*dIII are underlined). The PCR products were purified, treated with *Xba*I and *Hin*dIII, and then cloned into the *Xba*I and *Hin*dIII sites of pBAD24.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Reference
Strains		
LJ3001 (BW25113)	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	5
LJ3002 (BT340)	Δ(*argF-lac*)169 ϕ80*dlacZ58*(M15) *glnV44*(AS) λ$^-$ *rfbD1 gyrA96*(Nal^r) *recA1 endA1 spotT1 thi-1 hsdR17*	5
LJ3003 (BW25141)	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ Δ*phoBR580 hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ *galU95 endA*$_{BT333}$ *uidA*(Δ*Mlu*I)::*pir*$^+$ *recA1*	5
LJ3004 (BW25113ΔTBA)	BW25113Δ*sgaTBA*	This study
LJ3005 (BW25113ΔT)	BW25113Δ*sgaT*	This study
LJ3006 (BW25113ΔB)	BW25113Δ*sgaB*	This study
LJ3007 (BW25113ΔA)	BW25113Δ*sgaA*	This study
LJ3008 (JM101)	*supE thi* (Δ*lac-proAB*) *F′ traD36 lacI*^{q} *lacZ*ΔM15 *proAB*	8
LJ3009 (PB11)	JM101Δ*ptsH1 crr*::*km*	8
DH5α	*F*$^-$ *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*argE-lacZYA*)*169* ϕ80*lacZ*ΔM15	Gibco-BRL
LJ3015 (BW25113Δ*yjfQ*)	BW25113Δ*yjfQ*	This study
LJ3016 (BW25113Δ*yjfR*)	BW25113Δ*yjfR*	This study
LJ3017 (BW25113Δ*crp*)	BW25113Δ*crp*	This study
LJ3018 (BW25113Δ*fnr*)	BW25113Δ*fnr*	This study
LJ3019 (BW25113Δ*fur*)	BW25113Δ*fur*	This study
Plasmids		
pKD46	*oriR101 repA101*(*ts*) *araBp-gam-bet-exo ap*	5
pKD4	*oriR*$_Y$ Ap^r Km^r	5
pCP20	λ*cI*857(*ts*) *ts-rep*	5
pBAD24	Ap^r	12
pBAD24-*sgaTBA*	pBAD24 carrying the *sgaTBA* gene cluster that contains *sgaT*, *sgaB*, and *sgaA*	This study
pBAD24-*sgaT*	pBAD24 carrying *sgaT*	This study
pBAD24-*sgaB*	pBAD24 carrying *sgaB*	This study
pBAD24-*sgaA*	pBAD24 carrying *sgaA*	This study
pBAD24-*yjfQ*	pBAD24 carrying *yjfQ*	This study
pBAD24-*yjfR*	pBAD24 carrying *yjfR*	This study

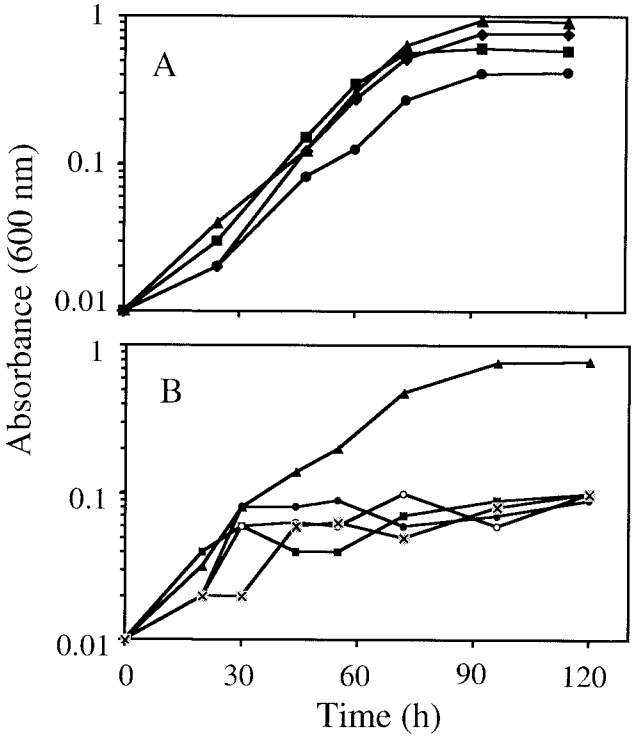

FIG. 2. Growth of *E. coli* strains on L-ascorbate. Growth was conducted in minimal medium M9 (24) under anaerobic conditions (see Materials and Methods) (A) Growth of wild-type *E. coli* as a function of time at various L-ascorbate concentrations. ■, 10 mM; ▲, 20 mM; ♦, 30 mM; ●, 50 mM. (B) Growth of wild-type and mutant strains in 20 mM L-ascorbate. ▲, wild type; ■, Δ*sgaTBA*; ●, Δ*sgaT*; ○, Δ*sgaB*; X, Δ*sgaA*.

In vivo transport assays. Transport studies were conducted essentially as described by Djordjevic et al. (6). Cells grown anaerobically in M9 medium were harvested during the logarithmic phase, washed three times with Tris-maleate (TM) buffer (pH 7.0), and resuspended in the same buffer containing 0.5% D,L-lactate as a source of energy. Uptake was conducted with cell suspensions (1 ml; OD_{600} = 2.0) in 1.5-ml microcentrifuge tubes with 30 μM L-ascorbate (5 μCi/μmol) unless otherwise noted. Aliquots (100 μl) were periodically removed, filtered through 0.45-μm-pore-diameter Millipore filters, washed three times with TM buffer, and dried. Radioactivity on the filters was measured by scintillation counting with 10 ml of Bio-safe NA fluid (Research Products Int. Corp., Mt. Prospect, Ill.). Values are expressed as picomoles of [^{14}C]L-ascorbate taken up per milligram (dry weight) of cells (see Fig. 7) or as micromoles of [^{14}C]L-ascorbate taken up per gram (dry weight) of cells per hour (see Fig. 8).

In vitro phosphorylation assays. Cells from 1.25 liters of fresh exponentially harvested cultures grown anaerobically in M9 medium supplemented with 20 mM L-ascorbate and 20 mM D-glucitol were washed four times at 4°C with the medium 63 salts (23), resuspended in 25 ml of 63 salts medium plus 5 mM dithiothreitol, and passed twice through the French press at 10,000 lb/in^2. The cell lysates were centrifuged (10,000 rpm for 1 min at 4°C), and the pellets were discarded. The resultant supernatants were centrifuged (13,000 rpm for 10 min), and the pellets were resuspended in 1.5 ml of the original crude enzyme extract. Using this procedure, the cell membranes were concentrated without removing the soluble enzymes of the PTS or exposing the membranes to a protein-free buffer. For standard phosphorylation assays, 200 μl of crude enzyme extract was added to 200 μl of 2× concentrated phosphoenolpyruvate (PEP)-dependent phosphorylation assay buffer containing 20 μM [^{14}C]L-ascorbate and 10 mM PEP essentially as described by Aboulwafa and Saier (1). After incubation at 37°C for 2, 5, or 10 h, the membrane pellet was removed from the assay mix by centrifugation, and 400 μl of 5 M $CaCl_2$ was added to the aspirated supernatant. The preparations were centrifuged (20,000 × *g* for 2 min), and the pellets were washed three times with 1 ml of 5 M $CaCl_2$ and then resuspended in 3 ml of water used in three successive 1-ml portions. Radioactivity in the $CaCl_2$ precipitate, including the calcium salt of L-ascorbate-6-P, but not of L-ascorbate, was measured by scintillation counting with 10 ml of Bio-safe II fluid (Research Products Intl. Corp., Mt. Prospect, Ill.). To demonstrate the dependency on PEP, various concentrations of this phosphoryl donor were included in the assay solution. A PEP concentration of 10 mM gave nearly maximal activity, and consequently this concentration was used routinely unless otherwise stated.

For phosphatase treatment, the membrane pellet was removed from the assay mix by centrifugation after completion of the phosphorylation reaction, and 25 U of either acid phosphatase or alkaline phosphatase was added prior to the addition $CaCl_2$. The pH was adjusted to 4.8 (acid phosphatase) or 9.8 (alkaline phosphatase) before addition of phosphatase, and the preparation was incubated for an additional 40 min before neutralization and addition of 5 M $CaCl_2$ to terminate the reaction and quantitate the pelletable radioactive phosphate ester. Both acid and alkaline phosphatase reduced the radioactive phosphorylated product formed and recovered in the $CaCl_2$ precipitate to background levels (<0.5% of the activity observed for the wild-type enzyme without phosphatase treatment). Protein concentrations were determined with the Bio-Rad colorimetric protein assay kit (catalog no. 500-0006) with bovine serum albumin as the standard protein.

Materials. 1-[^{14}C]L-ascorbate was purchased from Perkin-Elmer Life Sciences, Inc., and [^{14}C]mannitol and [^{14}C]L-proline were purchased from ICN Pharmaceuticals, Inc. (Irvine, Calif.). 2-Keto-L-gulonate and 2,5-diketo-L-gulonate were generously provided by Fernando Valle of Genencor International, Inc., Palo Alto, Calif. Acid and alkaline phosphatases (catalog no. P1146 and P6772, respectively), carbonyl cyanide-*m*-chlorophenylhydrazone (catalog no. C2759), Na arsenate (catalog no. A6756), and all other nonradioactive compounds were purchased from the Sigma Chemical Corp unless otherwise stated. All compounds were of the highest purity available commercially.

RESULTS

Growth studies. Figure 2A shows the growth of wild-type *E. coli* cells under anaerobic conditions with various concentrations of L-ascorbate as the sole source of carbon and energy. The optimal concentration of L-ascorbate under the conditions used was 20 mM. Both the growth rate and the extent of growth were optimal at this concentration (Fig. 2A). The doubling time with 20 mM L-ascorbate was 16.3 h, as compared with a doubling time with 20 mM D-glucose of 1.6 h. In a separate experiment, preinduced BW25113 cells (i.e., cells previously grown for 72 h in minimal L-ascorbate medium) were tested for the utilization of L-ascorbate in comparison to the nonpreinduced cells. Preinduction reduced the lag phase by about 20 h, but did not alter the growth rate or the growth yield. It was therefore concluded that slow induction at least in part accounts for the long lag period observed before growth ensues (Fig. 2A) (data not shown).

In-frame deletions in the coding regions of the *sgaTBA* gene cluster as well as each one of these three genes resulted in complete loss of L-ascorbate utilization (Fig. 2B). The effects of expression of the *sgaTBA* genes from a multicopy plasmid on L-ascorbate utilization by the wild-type and mutant strains were measured to determine whether these deletions could be fully or partially complemented by the *sgaTBA* gene cluster. pBAD24-*sgaTBA* (see Materials and Methods), when expressed in *trans*, increased the rate of growth of the wild-type strain on L-ascorbate (data not shown) (see Fig. S1 on our web site). The presence of the pBAD24-*sgaTBA* plasmid restored anaerobic growth on L-ascorbate to wild-type rates for all four mutants. The *sgaT*, *sgaB*, and *sgaA* mutants could also be complemented by inclusion of the pBAD24 plasmids expressing each of these genes individually (data not shown). It can therefore be concluded that these chromosomal mutations do not prevent L-ascorbate utilization by having a polar effect on the downstream genes encoding the metabolic enzymes (Fig. 1)

(31). The results consequently show that all three genes are required for growth on L-ascorbate.

In order to investigate the dependency of L-ascorbate utilization on the PTS energy-coupling enzymes, enzyme I and HPr, two isogenic strains, a wild-type strain, and a strain with the *pts* operon deleted *(ΔptsHIcrr::km)* (Table 1), were examined. The Δ*pts* strain could not utilize L-ascorbate, although the isogenic wild-type strain could (data not shown; see Fig. S2 on our web site). Addition of cyclic AMP to the growth medium at a concentration of 0.25, 0.5, 1.0, or 2.5 mM did not cause detectable growth of the mutant. These results show that the absence of the energy-coupling enzymes of the PTS prevented L-ascorbate utilization, and the exogenous addition of cyclic AMP did not restore growth. These observations suggest that enzyme I and HPr play a primary role in L-ascorbate utilization rather than a secondary role due to a regulatory effect on adenylate cyclase activity (20).

In vivo transport studies. Transport studies were conducted with [^{14}C]L-ascorbate as the radioactive substrate and under the conditions essentially described by Djordjevic et al. (6) (see Materials and Methods). When the sole source of carbon for growth was 20 mM L-ascorbate, uptake of the radioactive substrate was much greater than when cells were grown in glucose-containing medium (Fig. 3A). This fact suggests that the *sgaTBA* genes are inducible by the presence of L-ascorbate. When both carbon compounds were present in the growth medium, uptake was reduced by 40% (Fig. 3A). We therefore examined the mutants with the *sgaTBA* gene cluster or any one of these three *sga* genes deleted. Transport was reduced to negligible values, regardless of which mutation had been introduced (Fig. 3B). Since each deletion mutation is an in-frame deletion, and growth on L-ascorbate was restored by complementation with plasmid pBAD24-*sgaTBA* or with the plasmid bearing the deleted gene (described above), it can be concluded that to observe L-ascorbate uptake, the products of all three genes are required.

The experiments shown in Fig. 3B were repeated essentially as described therein, except that D-glucose was replaced by D-glucitol (sorbitol), a poorly catabolite-repressing PTS sugar. The results were essentially the same except, that uptake by the wild-type cells was reduced only 20% by the presence of 20 mM D-glucitol (data not shown). Thus, the lack of [^{14}C]L-ascorbate uptake by the mutants could not be explained by a phenomenon such as hypersensitivity to glucose repression. Because of the higher activity observed for glucitol/ascorbate-grown cells, this medium was used for growth of cells for subsequent transport studies and for the in vitro experiments reported in the next section.

Uptake of [^{14}C]L-ascorbate was examined in the presence of arsenate, which preferentially blocks ATP- or PEP-dependent uptake, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which preferentially blocks proton or sodium motive force-dependent uptake. As controls, uptake of [^{14}C]mannitol (PEP dependent) and [^{14}C]proline (sodium motive force dependent) was studied (13, 14, 16). With the ratio of the concentrations of arsenate and CCCP at 4,000 (i.e., 1 μM CCCP and 4 mM arsenate or 5 μM CCCP and 20 mM arsenate), proline uptake was always more strongly inhibited by CCCP, while mannitol uptake was always more strongly inhibited by arsenate. Inhibition of L-ascorbate uptake generally followed

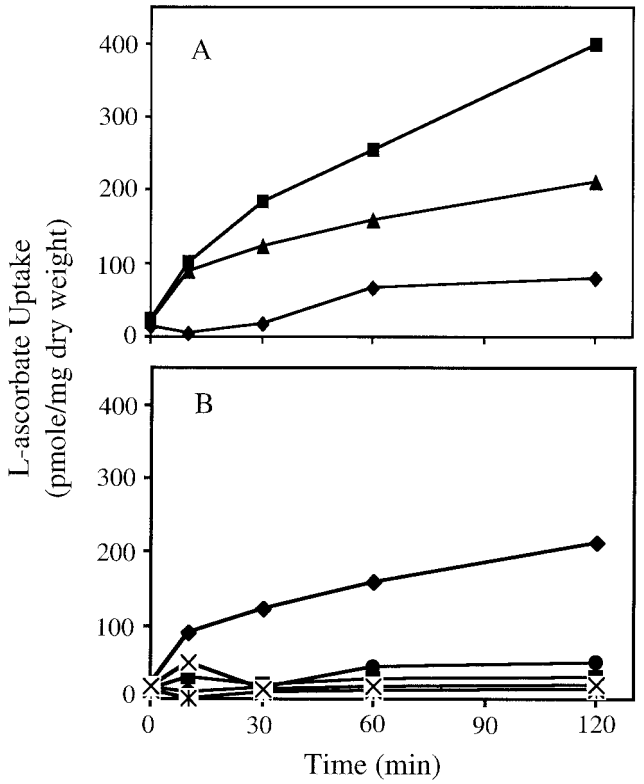

FIG. 3. Uptake of [^{14}C]L-ascorbate. In panel A, cells were grown anaerobically in medium M9 plus 20 mM L-ascorbate (■), 20 mM glucose (♦), or 20 mM L-ascorbate plus 20 mM glucose (▲). In panel B, the cells were grown in 20 mM L-ascorbate plus 20 mM glucose. ♦, wild type; ■, Δ*sgaTBA*; ●, Δ*sgaT*; ∗, Δ*sgaB*; X, Δ*sgaA*. Values are expressed as picomoles of [^{14}C]L-ascorbate retained by the cells per milligram (dry weight).

that of mannitol uptake, being stronger in the presence of arsenate than of CCCP (data not shown). These preliminary results provided the first evidence that a chemical form of energy rather than a chemiosmotic form of energy is coupled to L-ascorbate uptake. This conclusion was substantiated by the in vitro phosphorylation studies reported below.

The isogenic pair of *E. coli* strains lacking or possessing an intact *pts* operon was examined for L-ascorbate uptake following growth in medium containing L-ascorbate (20 mM) plus D-glucitol (20 mM). No uptake was observed for the mutant, although uptake was observed for the wild-type strain (data not shown) (see Fig. S3 on our web site).

In vitro phosphorylation studies. Phosphorylation of [^{14}C]L-ascorbate was examined by using crude extracts enriched for the pelleted membrane fraction as described in Materials and Methods. The results are presented in Fig. 4. A crude enzyme preparation from the wild-type strain (BW25113) exhibited substantial activity. The phosphorylated product was completely (>99%) lost (converted to a nonphosphorylated product) by addition of acid or alkaline phosphatase to the assay mixture after phosphoryl transfer from PEP to [^{14}C]L-ascorbate had occurred (see Materials and Methods). This result shows that the radioactive product is a phosphate ester. Minimal activity was observed when PEP was omitted from the assay mix or when the enzyme extract was derived from the

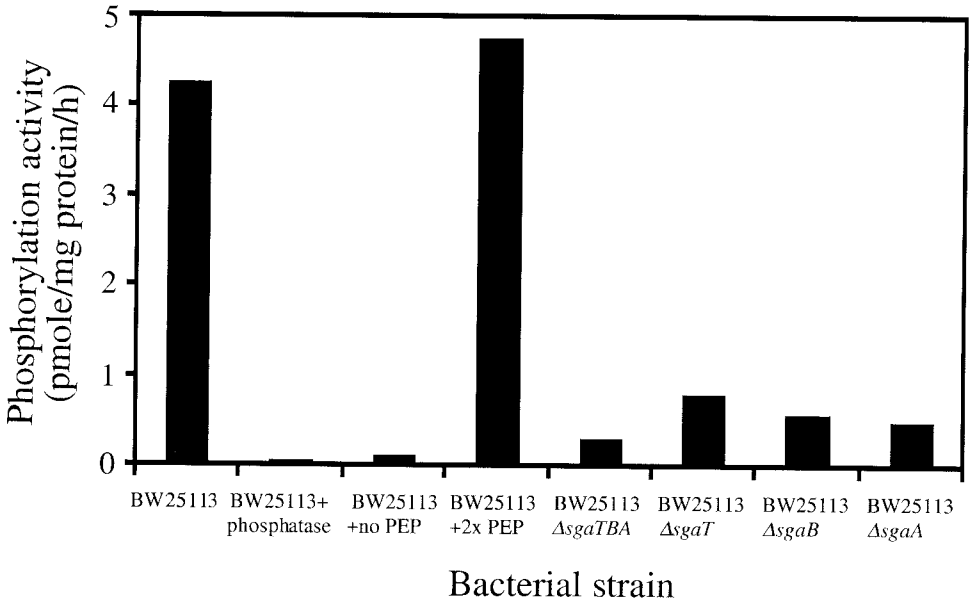

FIG. 4. In vitro phosphorylation of [^{14}C]L-ascorbate using PEP as the phosphoryl donor. The experiment was conducted as described in Materials and Methods. The addition of acid or alkaline phosphatase following assay of the wild-type extract for [^{14}C]L-ascorbate phosphorylation resulted in the complete loss of activity, and the Δ*sgaTBA* and single gene mutants exhibited greatly depressed activity as shown. The values are expressed as picomoles of L-ascorbate phosphorylated per milligram of protein per hour.

Δ*sgaTBA* mutant or any one of the single gene mutants (*sgaT*, *sgaB*, or *sgaA*) (Fig. 4). Increasing the PEP concentration from 10 mM to 20 mM increased phosphorylation activity observed with the wild-type extract by 15% (Fig. 4). These results show that the reaction is PEP dependent and that the phosphorylation activity observed for the wild-type strain is dependent on all three *sgaTBA* gene products. On the basis of both the in vivo transport studies reported above and the in vitro phosphorylation studies shown in Fig. 4, we conclude that SgaTBA comprises an enzyme II complex capable of phosphorylating L-ascorbate.

Regulatory studies identifying the YjfQ repressor. We examined the possibility that the upstream *yjfQ* gene, encoding a homologue of the DeoR repressor (15), might be a transcriptional regulator of the *sga* operon. The *yjfQ* gene was cloned into the pBAD24 vector, and the chromosomal gene was deleted (see Materials and Methods). Our results with this deletion strain and the wild-type strain bearing the overexpression plasmid were as follows. (i) Introduction of a Δ*yjfQ* mutation allowed more rapid utilization of L-ascorbate and substantially reduced the lag phase (Fig. 5A). (ii) This mutation also allowed growth of the cells on L-ascorbate under microaerophilic conditions, conditions under which the wild-type cells could not grow (Fig. 5B). (iii) Inclusion of a plasmid (pBAD24-*yjfQ*) overexpressing the *yjfQ* gene in both the wild-type and Δ*yjfQ* strains resulted in greatly depressed growth on L-ascorbate (Fig. 6). (iv) The *yjfQ* mutant exhibited enhanced uptake of [^{14}C]L-ascorbate following growth in the presence of L-ascorbate or D-glucitol as the sole carbon source (Fig. 7). These results show that YjfQ is a repressor of the *sga* operon and that it influences growth under microaerophilic conditions. These results agree with and extend the results of Campos et al. (3).

Dependency of L-ascorbate utilization on Crp and Fnr, but not Fur. *crp*, *fnr*, and *fur* deletion mutants were constructed in the genetic background of *E. coli* strain BW25113 as described in Materials and Methods (Table 1). The *crp* mutant was totally incapable of growth with 20 mM L-ascorbate under our standard anaerobic conditions (data not shown; see Fig. S4 on our web site). The *fnr* mutant showed greatly delayed growth

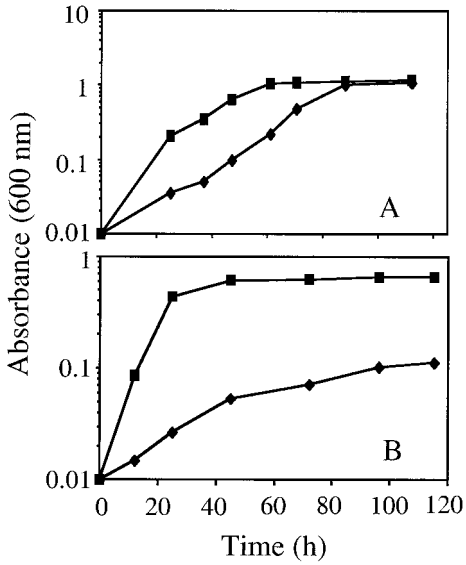

FIG. 5. Growth of isogenic wild-type and *yjfQ* deletion mutant strains under anaerobic (A) and microaerophilic (B) conditions. Growth in both cases was conducted with medium M9 plus 20 mM L-ascorbate. Anaerobic conditions (A) were as described under Materials and Methods. Microaerophilic conditions (B) resulted from the use of a loosely capped 16-mm-diameter tube containing 8 ml of medium with cells grown at 37°C without shaking. Aliquots were periodically removed for determination of the A_{600}. ◆, wild type; ■, Δ*yjfQ*.

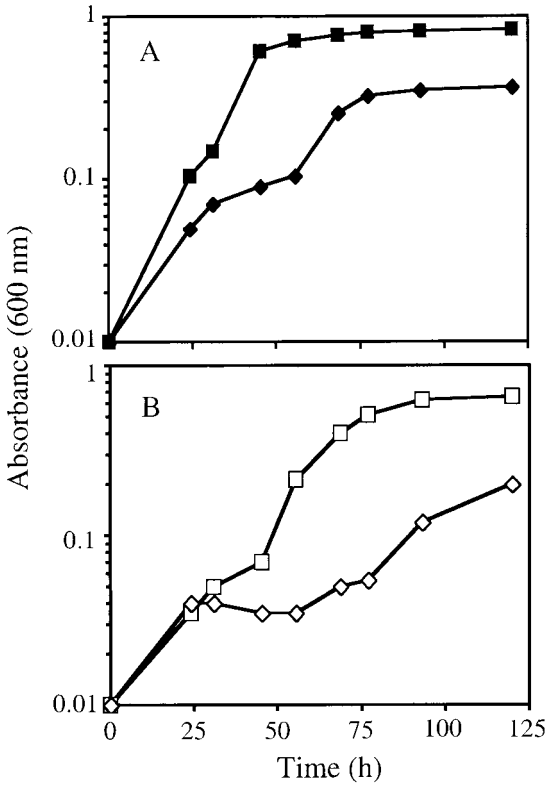

FIG. 6. Effect of a *yjfQ* deletion mutation and of *yjfQ* overexpression on L-ascorbate utilization. Anaerobic growth was conducted with 20 mM L-ascorbate plus 100 μg of ampicillin per ml as described in Materials and Methods. The following strains were used: wild type plus pBAD24 (♦) and Δ*yjfQ* mutant plus pBAD24 (■) in panel A and wild type plus pBAD24-*yjfQ* (◇) and Δ*yjfQ* plus pBAD24-*yjfQ* (□) in panel B.

under anaerobic conditions with L-ascorbate (20 mM) as the sole source of carbon (data not shown; see Fig. S5 on our web site). The *fur* mutant grew at the wild-type rate, although it reached the stationary phase at a 20% lower cell density. Five or 10 μM $FeCl_3$ did not alter the rate or extent of growth of the *fur* mutant, but 20 μM $FeCl_3$ had a slight inhibitory effect on the growth rate without altering the extent of growth (data not shown) (see Fig. S6 on our web site). These results suggest that transcription of the *sga* regulon is (i) under the control of Crp, (ii) influenced by Fnr, and (iii) not appreciably affected by Fur.

Essentiality of the *yjfR* gene product for L-ascorbate utilization. The *yjfR* gene, which encodes a distant homologue of a metal-dependent hydrolase, was cloned into pBAD24, and the chromosomal gene was deleted. The deletion strain was then compared with the wild-type strain and the wild-type strain overexpressing *yjfR* on the pBAD24 plasmid. The results were as follows. (i) The null *yjfR* mutant could not grow with L-ascorbate as the sole source of carbon (data not shown; see Fig. S7 on our web site). (ii) Exposure of L-ascorbate to acid (pH 2) or alkaline (pH 12) conditions for 12 h at 55°C did not promote growth (data not shown). (iii) The overexpressing strain grew better than the wild-type strain with an increased rate and increased extent of growth (20% increase of both) (data not shown; see Fig. S8 on our web site). (iv) The *yjfR* mutant appeared to take up [^{14}C]L-ascorbate at a much greater rate than the wild type, possibly because metabolism of the cytoplasmic radioactive product was prevented. (v) The transport K_m was depressed about twofold relative to the wild-type strain (see next section). (vi) Extracts containing YjfR could not hydrolyze L-ascorbate to 3-keto-L-gulonate (W. S. Yew, Z. Zhang, M. H. Saier, Jr., and J. Gert, unpublished results). (vii) No N-terminal signal sequence targeting YjfR to the periplasm could be identified. These results suggest that YjfR is a cytoplasmic L-ascorbate-6-phosphate lactonase, which is essential for L-ascorbate utilization.

Affinity of the SgaTBA permease for L-ascorbate. The uptake of L-ascorbate was studied as a function of L-ascorbate concentration both in the wild-type strain and in the *yjfR* mutant. The results for the *yjfR* mutant are shown in Fig. 8. The K_m value calculated from the plot was 9 μM, while the V_{max} was 1 μmol/g (dry weight) of cells per hour (see insert to Fig. 8). When the corresponding data for the wild-type strain were analyzed, a value of 18 μM was obtained for the K_m, and the V_{max} was much lower (data not shown; see Fig. S9 on our web site). Since uptake in the wild-type strain represents a sum of transport, metabolism, and product excretion, the latter value is not likely to reflect the transport process alone. We suggest that the most reliable K_m value for L-ascorbate uptake by the SgaTBA PTS permease is 9 μM.

Inhibitory effects of L-ascorbate analogues and various sugars. We tested the effects of L-ascorbate; the two L-ascorbate analogues, 2-ketogulonate and 2,5-diketogulonate; as well as 16 hexoses, pentoses, hexitols, and pentitols for inhibition of L-ascorbate uptake with the radioactive substrate present at a concentration of 50 μM (the wild-type strain) or 20 μM (the *yjfR* mutant strain), and the inhibitory compound was present in 10-fold excess (500 or 200 μM, respectively). Nonradioactive L-ascorbate itself inhibited 72% ± 5%. All other analogues inhibited <15%, except for D-arabitol, which inhibited 34% ± 8% (data not shown; see Table S3 on our web site). We conclude that the L-ascorbate transporter is highly specific for L-ascorbate.

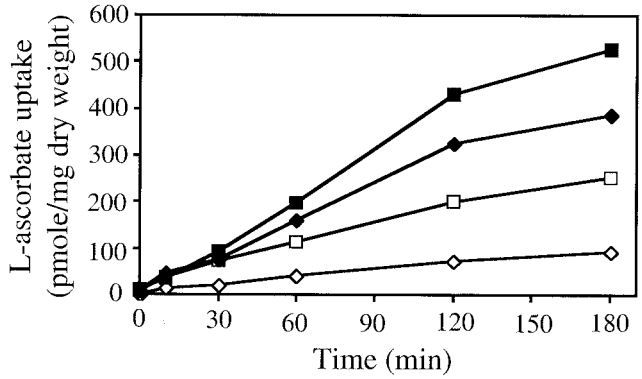

FIG. 7. Uptake of [^{14}C]L-ascorbate by wild-type cells (diamonds) and the Δ*yjfQ* mutant (squares). Solid symbols represent induced conditions (growth with 20 mM L-ascorbate), and open symbols represent uninduced conditions (growth with 20 mM D-glucitol).

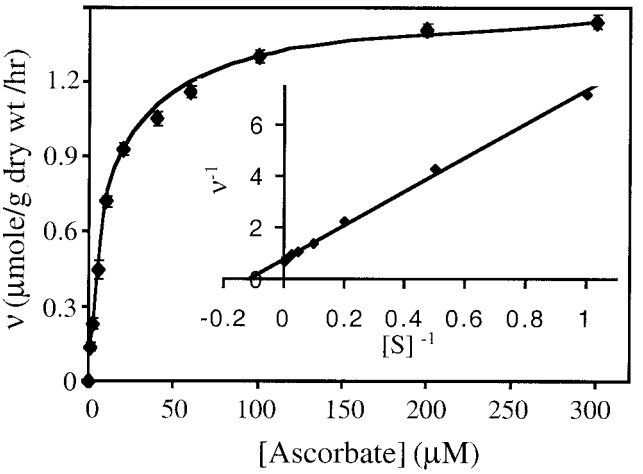

FIG. 8. Uptake of [^{14}C]L-ascorbate by the Δ*yjfR* mutant as a function of L-ascorbate concentration (1 to 300 μM concentration range). The uptake experiment was conducted as described in Materials and Methods after anaerobic growth in 20 mM L-ascorbate plus 20 mM D-glucitol. The inset shows a double-reciprocal plot of the data.

DISCUSSION

The results presented in this paper establish that all three gene products, SgaA, SgaB, and SgaT, are required for L-ascorbate utilization, for L-ascorbate uptake in vivo, and for L-ascorbate phosphorylation in vitro. Together, SgaABT presumably comprise the L-ascorbate PTS permease as well as the L-ascorbate phosphotransferase. The conclusion that three gene products are required for L-ascorbate uptake and phosphorylation is consistent with the suggestion that SgaT, SgaB, and SgaA function together as a novel type of enzyme II complex, where SgaT is equivalent to previously characterized enzymes IIC and where SgaA and SgaB, which are homologous to known enzymes IIA and IIB (see above) (18, 26), serve as the sugar-specific energy-coupling phosphoryl transfer proteins of the ascorbate-specific PTS. This is the first instance in which an enzyme IIC exhibits the topology of a typical 12 TMS carrier, and also the first incidence where the sugar-specific IIA and IIB proteins are derived from different families: i.e., IIA^{SgaA} is in the fructose/mannitol family (transport classification [TC] # 4.A.2), while IIB^{SgaB} is in the lactose *N*,*N*′-diacetylchitobiose family (TC # 4.A.3) (21, 22). The presumed phosphoryl transfer reaction responsible for L-ascorbate transport and phosphorylation is therefore:

$$\text{PEP} \rightarrow \text{EI} \rightarrow \text{HPr} \rightarrow \text{SgaA} \rightarrow \text{SgaB} \xrightarrow[\text{ascorbate (out)}]{\text{SgaT}} \text{ascorbate-6-P (in)}$$

Homologues of SgaT, like other PTS protein homologues, have been identified in a large number of evolutionarily divergent bacteria, but not in archaea or eukaryotes (M. H. Saier, Jr., unpublished results). Bacteria which encode SgaT homologues include numerous gram-negative proteobacteria, as well as many low- and high-G+C gram-positive bacteria. Except for species of *Corynebacterium*, *Streptomyces*, and *Bacillus*, almost all organisms possessing SgaTBA homologues are human or animal pathogens. Several organisms have two or more SgaT paralogues, including *E. coli*, which has three. In *E. coli*, our results suggest that the SgaTBA homologues do not transport L-ascorbate, since the *sgaA*, *sgaB*, and *sgaT* mutants are negative for L-ascorbate utilization, uptake, and phosphorylation. In some of the homologues found in other bacteria, SgaB domains are fused C terminal to the SgaT domains. For example, this is true of putative transporters in *Vibrio cholerae* (AAP96157; 586 amino acids [aa]), *Pasteurella multocida* (AAK02848; 625 aa), and *Mycoplasma pulmonis* (CAC13371; 650 aa). Homologues of SgaB and SgaA, but not SgaT, are also found in transcriptional activator proteins, where they function in regulation rather than sugar transport (10). A detailed bioinformatic analysis of SgaTBA systems will be published elsewhere.

The identification of SgaTBA as a novel enzyme II complex of the PTS represents an important advance, since SgaT is not homologous to any previously characterized enzyme IIC of the PTS. It does not, in fact, exhibit the topological features of any other recognized enzyme IIC. Topologically, it more closely resembles secondary carriers, although it is not homologous to any known secondary carrier. The recent discovery of a nonhomologous, nontransporting enzyme II complex specific for dihydroxyacetone resembling in sequence functionally characterized ATP-dependent dihydroxyacetone kinases (11) illustrates the versatility of the PTS in recruiting proteins that evolved for other catalytic purposes into this PEP-dependent PTS system. Since not all established enzyme II complexes are homologous (22), the use of SgaT as an enzyme IIC of the PTS, while representing a unique and novel example, does not establish a new principle. Nevertheless, the mechanism of phosphoryl transfer from SgaB-P to the substrate sugar acid may well prove to exhibit unique features. Further work will be required to establish the mechanistic details.

E. coli and several other enteric bacteria are normal inhabitants of the mammalian intestine, an organ that is essentially anaerobic. Because L-ascorbate is a common constituent of many plant and animal tissues (as well as of vitamin C tablets), the knowledge that enteric bacteria can transport and metabolize L-ascorbate under anaerobic conditions suggests that intestinal bacteria can compete with the intestinal mucosal cells for available sources of vitamin C. Mammals possess two high-affinity, stereospecific, L-ascorbate:Na^+ symporters, one of which, SVCT1 (TC # 2.A.40.6.1), is localized primarily to the epithelial cells of the intestine, kidney, and liver (27). The K_m of this system for its substrate has been reported to be 75 to 250 μM (4, 29). Since the L-ascorbate PTS permease transports its substrate with low micromolar affinity (9 μM; 10-fold lower than that observed for the mammalian system), bacterial uptake systems should effectively compete with the mammalian intestinal transporter. The fact that the *E. coli* system is only induced under anaerobic conditions fits with the anaerobic environment that exists in the intestinal lumen. The high substrate affinity displayed by the bacterial L-ascorbate permease is in the same range as that estimated from K_m values reported for other PTS permeases (17). The physiological consequences of the competitive use of ascorbate by intestinal bacteria to human and animal health have yet to be evaluated. It will be interesting to determine what fraction of L-ascorbate enters bacteria versus the intestinal mucosal cells under a variety of normal physiological conditions in vivo.

ACKNOWLEDGMENTS

We thank Guillermo Gosset for providing the isogenic pair of *E. coli* strains, JM101 and PB11; Fernando Valle of Genencor International, Inc., for providing 2-keto-L-gulonate and 2,5-diketo-L-gulonate; John Gerlt and Wen Shan Yew for critically reading the manuscript; and Mary Beth Hiller for assistance in its preparation.

This work was supported by NIH grant no. GM55434.

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